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(54) Title: IMMUNOSTIMULATION MEDIATED BY GENE-MODIFIED DENDRITIC CELLS

(57) Abstract

Compositions and methods useful for stimulating an immune response against one or more disease associated antigens by genetically modifying dentritic cells *in vivo* or *ex vivo* are provided. These compositions and methods allow for administration of lower dosages of gene delivery vehicles in order to achieve levels of immune stimulation comparable to those obtainable by conventional methods. Alternatively, administration of conventional dosages of gene delivery vehicles will enhance the resultant immune response.

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Immunostimulation Mediated By Gene-Modified Dendritic Cells

Technical Field of the Invention

The present invention relates generally to recombinant DNA technology. In particular, the invention concerns compositions and methods useful for the prophylactic or therapeutic stimulation of the immune system of an animal by (i) *in vivo* transduction of dendritic cells or (ii) administration of dendritic cells transduced *ex vivo* with an expression vector functionally encoding at least one disease associated antigen.

Background of the Invention

Immune system stimulation to antigens associated with disease is an accepted approach to disease prevention. Traditional techniques have involved use of killed or attenuated-live vaccines made from various viral pathogens. The advent of recombinant DNA technology has enabled development of a new, more safe generation of vaccines to combat viral infections, wherein the immune stimulant is typically an immunogenic protein encoded by the pathogen.

Despite these technologies, few effective treatments or prophylactic measures have been developed for many viral diseases and cancer. Recently, several groups have reported immune induction in animals against HIV-encoded gene products through the use of gene therapy technology. Specifically, autologous fibroblasts transduced *ex vivo* with a retroviral vector encoding the HIV env/rev genes were injected into mice, non-human primates, and humans lead to induction of an immune response against these antigens. *See* Warner, *et al.*, 1991; Laube, *et al.*, 1993; and Zeigner, *et al.*, 1994.

Such *ex vivo* approaches, however, are not practical for large scale vaccination programs, as a separate product, *i.e.*, transduced autologous cells, must be generated for each patient. To overcome this and other shortcomings of *ex vivo* approaches, efforts are being undertaken to develop *in vivo* techniques wherein a gene delivery vehicle carrying an expression vector which directs expression of a disease-specific immunogen is administered directly to a patient. *See* WO 91/02805, WO 93/10814, WO 93/15207, WO 94/06921, WO 94/21792, and WO 95/07994. Irwin, *et al.* (!994) reported immune

induction against specific immunogens following intramuscular injection of recombinant retroviruses in mice, rhesus monkeys, and baboons. Compositions and methods which enable improved immune stimulation against disease associated antigens are needed.

Summary of the Invention

It is the object of this invention to provide compositions and methods for the immunoprophylactic or immunotherapeutic treatment of animals, including mammals, particularly humans. The compositions of the invention can be used to deliver an immunogen, *i.e.*, a disease associated antigen, to an animal in order to immunize it against disease, such as cancer or, alternatively, against bacterial, parasitic, or viral infections. Such immunization results from generation of a cell mediated immune response against the immunogen(s) (i) encoded by the expression vector delivered by a gene delivery vehicle which are then expressed and presented on the surface of an antigen presenting cell (APC) *in vivo* or (ii) presented on the surface of an APC, particularly a dendritic cell, transduced *ex vivo* by such an expression vector.

One aspect of the invention relates to gene delivery vehicles targeted to dendritic cells, be they *in vivo* or *in vitro*. Such gene delivery vehicles comprise a dendritic cell targeting element and an expression vector which directs expression of at least one disease associated antigen. In one embodiment, the expression vector is carried by a recombinant virus. Recombinant viruses useful in the practice of the invention include both DNA and RNA viruses. In preferred embodiments, the recombinant virus is one derived from either a negative strand RNA virus or a positive strand RNA virus. Representative positive strand RNA viruses from which recombinant viruses can be derived include retroviruses (*e.g.*, avian leukosis virus, bovine leukemia virus, murine leukemia virus, mink-cell focus-inducing virus, murine sarcoma virus, reticuloendotheliosis virus, rous sarcoma virus, Mason-Pfizer monkey virus, baboon endogenous virus, endogenous feline retrovirus, gibbon ape leukemia virus, HIV I, HTLV I, and HTLV III), particularly murine retroviruses, togaviruses, *e.g.*, alphaviruses, particularly Sindbis virus, Semliki Forest virus, and Venezuelan Equine Encephalitis virus, picornaviruses, and coronaviruses, with those derived from retroviruses and alphaviruses being preferred. Representative negative strand RNA viruses from which recombinant viruses can be derived include rhabdoviruses

(*e.g.*, vesicular stomatitis virus), myxoviruses, paramyxoviruses, orthomyxovirses (*e.g.*, influenza virus), and bunyaviruses. Useful DNA viruses from which recombinant viruses useful in practicing the invention may be derived include adenoviruses and adenoassociated viruses.

In other embodiments, the gene delivery vehicle is non-viral gene delivery vehicle, *i.e.*, the expression vector is not carried by a virus. The expression vector will be either DNA or RNA, and can be linear or circularized. A particularly preferred expression vector is a eukaryotic layered vector initiation system. In one embodiment, the expression vector is complexed with one or more polynucleotide condensing agents. Polynucleotide condensing agents include polycations. Preferred polycations include polylysine, polyarginine, histones, protamines, spermidine, and spermine, with polylysine being particularly preferred. In another embodiment, the expression vector is complexed only with the dendritic cell targeting element. In yet another embodiment, the expression vector is associated with lipids, preferably being encapsulated in liposomes, particularly liposomes made of cationic lipids.

When a gene delivery vehicle is targeted to a dendritic cell, the dendritic cell targeting element can be any molecule which targets the gene delivery vehicle to a dendritic cell. Various embodiments include those wherein the dendritic cell targeting element is selected from the group consisting of a high affinity binding pair, an antibody reactive against a dendritic cell surface marker, and an antigen binding domain derived from an antibody reactive against a dendritic cell surface marker. Preferred high affinity binding pairs include those selected from the group consisting of biotin/avidin, cytostatin/papain and phosphonate/carboxypeptidase A. Preferred dendritic cell surface markers, against which antibodies (or antigen binding domains derived therefrom) can be generated to produce dendritic cell targeting elements, include CD 11c, CD 54, CD 58, CD 25, CD 11a, CD 23, CD 32, CD 40, CD 1, CD 45, MHC Class I, MHC Class II, Mac-1, Mac-2, and Mac-3. In another embodiment, the dendritic cell targeting element is a hybrid envelope protein, wherein the envelope portion is derived from a viral envelope protein, *e.g.*, a retroviral envelope protein, and the dendritic cell targeting element is derived from a protein which specifically interacts with a molecule presented on a dendritic cell plasma membrane.

Expression vectors according to the invention direct expression of at least one disease associated antigen. Such antigens are preferably associated with diseases selected from the group consisting of cancer, a hyperproliferative disease, a bacterial infection, a parasitic infection, and a viral infection. The cancers that may be treated, inhibited, or prevented using the immunostimulatory compositions and methods described herein include breast cancer, colon cancer, melanoma, lung cancer, brain cancer, and leukemia, among others. Bacterial infections which can be treated, inhibited, or prevented include pneumonia, sepsis, tuberculosis, and staph infections, among others. Parasitic infections which can be treated include those which cause malaria (caused by protozoa of the genus *Plasmodium*, and include *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax*), sleeping (caused by trypanosomes), and river blindness, among others. Viral infections which may be treated, inhibited, or prevented using the compositions and methods described herein include those caused by hepatitis A, hepatitis B, hepatitis C, non-A, non-B hepatitis, hepatitis delta agent, CMV, Epstein-Barr virus, HTLV I, HTLV II, and HIV I, among others.

In one embodiment, the expression vector codes for a single disease associated antigen. In other embodiments, the expression vector codes for multiple disease associated antigens, such as 2, 3, 4, 5, 6, 7, 8, 9, or 10 or more antigens. When multiple disease associated antigens are encoded by a single expression vector, the antigens may be associated with the same or different diseases. Alternatively, compositions comprising combinations of expression vectors, each encoding for one or more antigens associated with a disease different from those encoded by other expression vectors, may also be prepared. Compositions encoding for one or more antigens associated with different diseases are particularly useful as vaccines

In yet another embodiment of this aspect of the invention, the expression vector also encodes an immunomodulatory cofactor.

Preferred embodiments of the invention relate to pharmaceutical compositions comprising the various gene delivery vehicles of the invention and a pharmaceutically acceptable carrier or diluent. Particularly preferred embodiments include those wherein the pharmaceutical compositions are in solid form.

A second aspect of the invention concerns *in vivo* methods for producing genetically modified dendritic cells. Such methods comprise administering to an animal a gene delivery vehicle targeted to a dendritic cell, wherein the gene delivery vehicle comprises a dendritic cell targeting element and an expression vector which directs expression of at least one disease associated antigen.

In a related aspect, methods of prophylaxis are provided. In one embodiment, prophylaxis is achieved by administering to an animal a prophylactically effective amount of a gene delivery vehicle according to the invention. In another embodiment, prophylaxis is achieved by administering to an animal a prophylactically effective amount of a dendritic cell population transduced *ex vivo* with a gene delivery vehicle carrying an expression vector encoding genetic information sufficient to direct expression of a gene encoding at least an antigenic portion of a disease associated antigen.

In yet another related aspect of the invention, methods are provided for the therapeutic treatment, not prophylaxis, of disease. In one embodiment, therapeutic treatment is achieved by administering to an animal a therapeutically effective amount of a gene delivery vehicle targeted to a dendritic cell, wherein the gene delivery vehicle carries an expression vector encoding genetic information sufficient to direct expression of a gene encoding at least an antigenic portion of a disease associated antigen. In another embodiment, therapeutic treatment is achieved by administering to an animal a therapeutically effective amount of a dendritic cell population transduced *ex vivo* with a gene delivery vehicle carrying an expression vector encoding genetic information sufficient to direct expression of a gene encoding at least an antigenic portion of a disease associated antigen.

Such methods of prophylaxis or treatment of an animal can be accomplished by a single direct injection of gene delivery vehicles or a dendritic cell population transduced *ex vivo* at a single time point or multiple time points. In another embodiment, the method comprises administration of gene delivery vehicles of the invention or an *ex vivo* transduced dendritic cell population nearly simultaneously to multiple sites. Preferred routes of administration include the intravenous and subcutaneous routes. Preferred animals for prophylactic treatment by such methods include birds, mammals, and fish.

Particularly preferred mammals include those selected from the group consisting of human, bovine, equine, canine, feline, porcine, and ovine animals.

Yet another aspect of the invention relates to *ex vivo* methods of producing genetically modified dendritic cells. In one embodiment, such methods comprise obtaining a population of cells comprised substantially of dendritic cells, *i.e.*, greater than about 50% dendritic cells, more preferably greater than about 75% dendritic cells, more preferably still greater than about 90% dendritic cells, with greater than about 95% dendritic cells being particularly preferred, and genetically modifying that population of cells through introduction of a gene delivery vehicle according to the invention. In another embodiment, the method comprises obtaining a first population of cells containing dendritic cells, genetically modifying the first population of cells through introduction of a gene delivery vehicle according to the invention, and isolating a second population of cells comprised substantially of dendritic cells from the genetically modified first population of cells in part. Preferred methods for isolating cell populations comprised substantially of dendritic cells include affinity chromatography and FACS.

Brief Description of the Drawings

Figure 1 illustrates some of the cell surface markers useful in identifying dendritic cells. Markers indicated with a (-) are not present on dendritic cells, and thus may be used in negative selection strategies. Those marked (+) are present on the dendritic cell surface, with (++) and (+++) indicating particularly useful dendritic cell markers. The (+), (++), and (+++) dendritic cell markers also represent suitable targets to which gene delivery vehicles according to the invention can be targeted.

Figure 2 is a flow chart depicting a splenocyte separation strategy using percoll density gradients.

Figure 3 illustrates the effect of dendritic cell therapy on CT26.p24. Balb/c mice were injected with a total of 2×10^5 tumor cells. On day 8,15, and 22, animals were treated as in the figure. The tumor volume of the mice was measured twice a week.

Figure 4 illustrates the effect of dendritic cell therapy on JC.p24. Balb/c mice were injected with a total of 3×10^5 tumor cells. On day 8,15, and 22, animals were treated as in the figure. The tumor volume of the mice was measured twice a week.

Figure 5 shows the effect of dendritic cell immunization on JC.p24. Balb/c mice were immunized with splenic dendritic cells transduced with p24 twice (one week apart) followed by injection of 3 x 10^5 tumor cells. The tumor volume of the mice was measured twice a week.

Figure 6 shows the effect of dendritic cell immunization on JC.p24 following a single immunization of BM-DC.p24. Balb/c mice were immunized once with BM-DC.p24 One week later, they were injected with a total of 3 x 10^5 tumor cells. The tumor volume of the mice was measured twice a week.

Figure 7 shows the effect of dendritic cell immunization on JC.p24. Balb/c mice were treated with D2SC/1.beta-gal and D2SC/1.p24 on days 1 and 8. They were subsequently injected with a total of 3 x 10^5 tumor cells on day 15. The tumor volume of the mice was measured twice a week.

Figure 8 illustrates the effect of dendritic cell immunization on CT26.p24. Balb/c mice were immunized on days 1 and 7 with the vaccines provided in the figure legend. On day 15, animals were injected with a total of 2 x 10^5 tumor cells. The tumor volume of the mice was measured twice a week.

Figure 9 shows comparisons between methods for antigen introduction to dendritic cells. This compares the retroviral vector vs protein or peptide loading. Balb/c mice were immunized on days 1 and 7 with the vaccines provided in the figure legend. On day 8, animals were injected with a total of 2 x 10^5 CT26.beta-gal tumor cells. The tumor volume of the mice was measured twice a week.

Definition of Terms

The following terms are used throughout the specification. Unless otherwise indicated, these terms are defined as follows:

"Gene delivery vehicle" refers to a construct which is capable of delivering, and, within preferred embodiments expressing, one or more gene(s) or sequence(s) of interest in a host cell. Representative examples of such vehicles include viral vectors, naked DNA or RNA expression vectors, DNA or RNA expression vectors associated with cationic condensing agents, DNA or RNA expression vectors encapsulated in liposomes, and certain eukaryotic cells (*e.g.*, producer cells). Within preferred embodiments of the

invention, a gene delivery vehicle includes a targeting element, *e.g.*, a member of a high affinity binding pair covalently linked to, expressed on, or included as an integral part of the exterior of the gene delivery vehicle.

"High Affinity Binding Pair" refers to a set a molecules which is capable of binding one another with a K_D of less than $10^{-y}M$, wherein y is selected from the group consisting of 8, 9, 10, 11, 12, 13, 14 and 15. As utilized herein, the "K_D" refers to the disassociation constant of the reaction A + B = AB, wherein A and B are members of the high affinity binding pair. As understood in the art, as the affinity of the two molecules increases, K_D decreases. Affinity constants may be readily determined by a variety of techniques, including, for example, by Scatchard analysis (*see* Scatchard, *Ann. N.Y. Acad. Sci. 51*:660-672, 1949). Representative examples of suitable affinity binding pairs include biotin/avidin, cytostatin/papain and phosphonate/carboxypeptidase A.

"Targeting element" refers to a molecule which is capable of specifically binding a dendritic cell. Within the context of this invention, a targeting element is considered to specifically bind a dendritic cell when a biological effect is seen in that cell type after binding of the targeting element and its complement, or, when there is greater than a 10 fold difference, and preferably greater than a 25, 50 or 100 fold difference between the binding of the coupled targeting element to dendritic cells and non-target cells. Generally, it is preferable that the targeting element bind to dendritic cells with a K_D of less than $10^{-5}M$, preferably less than $10^{-6}M$, more preferably less than $10^{-7}M$, and most preferably less than $10^{-8}M$ (as determined by Scatchard analysis, *supra*). In addition, when a high affinity binding pair is used for targeting, the targeting element will preferably bind dendritic cells with an affinity of at least 1 log (*i.e.*, 10 times) less than the affinity constant of the high affinity binding pair. Suitable targeting elements are preferably non-immunogenic, not degraded by proteolysis, and not scavenged by the immune system. Particularly preferred targeting elements preferably have a half-life (in the absence of a clearing agent) in an animal of between 10 minutes and 1 week. Representative examples of suitable targeting elements are set forth below in more detail.

"Targeting dendritic cells" refers to methods that target dendritic cells. Such methods are defined as follows: the gene delivery vehicle possesses an element that

causes increased transduction of dendritic cells following administration in any of a number of conventional ways, as compared to transduction that occurs when the gene delivery vehicle lacks the element; or the gene delivery vehicle is administered in a particular fashion or by a particular route so that transduction of dendritic cells is enhanced as compared to normal or conventional routes of administration.

"Clearing agent" refers to a molecule which binds and/or cross-links circulating, coupled targeting elements. Preferably, the clearing agent is non-immunogenic, specific to the coupled targeting element, and large enough to avoid rapid renal clearance. In addition, the clearing agent is preferably not degraded by proteolysis, and not scavenged by the immune system. Particularly preferred clearing agents include those which bind to the coupled targeting element at a site other than the affinity binding member, and most preferably, which bind in a manner that blocks the binding of the targeting element to its target. Numerous cleaving agents may be utilized within the context of the present invention, including for example those described by Marshall et al. in *Brit. J. Cancer* *69*:502-507, 1994.

"Expression Vector" refers to a recombinant nucleic acid molecule (DNA or RNA) capable of directing expression of one or more genes encoding a disease associated antigen. The expression vector must include a promoter (unless the expression vector is designed for position-specific integration adjacent to a functional promoter) operably linked to the antigen-encoding gene(s), and a polyadenylation sequence. In certain embodiments, the expression vector is part of a plasmid construct. In addition to the expression vector components, the plasmid construct may also include one or more of the following: a bacterial origin of replication; one or more selectable markers; a signal which allows the plasmid construct to exist as single-stranded DNA (*e.g.*, a M13 origin of replication); a multiple cloning site; and a "mammalian" origin of replication (*e.g.*, a SV40 or adenovirus origin of replication). In other embodiments, the expression vector is a recombinant viral genome, and will be either RNA or DNA, depending on the particular viral system being utilized. Alternatively, the expression vector may comprise *in vitro* transcribed RNA. As used herein, "expression vector" also refers to a vector which, after introduction into a cell, is converted to a different form. For example, the RNA genome carried a recombinant retrovirus is reverse transcribed into DNA and integrated into the

genome of the cell. For purposes of this invention, both RNA and DNA forms are "expression vectors."

"Altered Cellular Component" refers to proteins and other cellular constituents which are either associated with rendering a cell tumorigenic, or are associated with tumorigenic cells in general but are not required or essential for rendering the cell tumorigenic. Before alteration, the cellular components may be essential to normal cell growth and regulation, and include for example, proteins which regulate intracellular protein degradation, transcriptional regulation, cell-cycle control, and cell-cell interaction. After alteration, the cellular components no longer perform their usual regulatory functions, and hence the cell may experience uncontrolled growth. Representative examples of altered cellular components include ras*, p53*, Rb*, altered protein encoded by the Wilms' tumor gene, ubiquitin*, mucin*, protein encoded by the DCC, APC, and MCC genes, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, platelet derived growth factor (PDGF) receptor, insulin receptor, epidermal growth factor (EGF) receptor, and the colony stimulating factor (CSF) receptor. These as well as other cellular components are described in more detail below, as well as discussed in cited references.

"Non-tumorigenic" refers to altered cellular components which will not cause cellular transformation or induce tumor formation in nude mice. Representative assays which distinguish tumorigenic cellular components from non-tumorigenic cellular components are described in more detail below.

"Immunogenic" as utilized within the present invention refers to altered cellular components which are capable, under the appropriate conditions, of causing an immune response. This response must be cell-mediated and may include a humoral response. Representative assays which may be utilized to determine immunogenicity are described in more detail below.

Numerous aspects and advantages of the invention will be apparent to those skilled in the art upon consideration of the following detailed description which provides illumination of the practice of the invention.

Detailed Description of the Invention

The present invention is based on the discovery that expression and cell surface presentation of at least one disease associated antigen in dendritic cells can be used to generate a prophylactic or therapeutic immune response against the disease with which the antigen is associated. Moreover, it has been discovered that the efficiency of immune system stimulation mediated by genetically modified dendritic cells can be several orders of magnitude greater than that mediated by genetically modified fibroblasts, muscle, and other cell types. As a result, these discoveries enable improved approaches to gene therapy-mediated immune stimulation, either by reducing the dosage(s) required to achieve the desired prophylactic or therapeutic result or by enhancing immune responses.

Diseases suitable to treatment using an immunostimulation strategy include: viral infections, such as those caused by HBV (*see* WO 93/15207), HCV (*see* WO 93/15207), HPV (*see* WO 92/05248, WO 90/10459, EPO 133,123), Epstein-Barr Virus (*see* EPO 173,254; JP 1,128,788; and U.S. Patent Nos. 4,939,088 and 5,173,414), Feline Leukemia Virus (*see* WO 93/09070, EPO 377,842, WO 90/08832, and WO 93/09238), Feline Immunodeficiency Virus (U.S. Patent No. 5,037,753, WO 92/15684, WO 90/13573, and JP 4,126,085), HTLV I and II, and HIV (*see* WO 91/02805); cancers, such as melanoma, cervical carcinoma, colon carcinoma, renal carcinoma, breast cancer, ovarian cancer, prostate cancer, leukemias; and heart disease.

Dendritic cells are a system of antigen presenting cells that function to initiate immune responses (Steinman, R. (1991), *Annu. Rev. Immunol.*, *vol.* 9:272-296; *see also Research in Immunology*, *vol.* 140, *International Reviews in Immunology*, *vol.* 6, *Advances in Immunology*, *vol.* 47, and *Epidermal Langerhans Cells*, ed. G. Schuler). Dendritic cells are found in many non-lymphoid tissues but can migrate via the afferent lymph or the blood stream to the T-dependent areas of lymphoid organs. In non-lymphoid organs, dendritic cells include Langerhans cells and interstitial dendritic cells. In the lymph and blood, they include afferent lymph veiled cells and blood dendritic cells, respectively. Dendritic cells are not found in the efferent lymph. In lymphoid organs, they include lymphoid dendritic cells and interdigitating cells. As used in the context of this invention, each of these cell types and their progenitors shall be referred to as "dendritic cells," unless otherwise specified.

Dendritic cells have an unusual dendritic shape, are motile, and efficiently cluster and activate T cells that are specific for cell surface stimuli. Typically, dendritic cells in non-lymphoid organs, such as Langerhans cells and interstitial cells, become veiled cells (cells which continually extend and retract large lamellipodia) in the afferent lymph and blood which migrate to lymphoid tissues, where they can be isolated as dendritic or interdigitating cells.

Partially enriched populations of epidermal Langerhans cells, wherein Langerhans cells may comprise up to about 60% of the total cell population, may be readily prepared, since keratinocytes can be depleted from murine tissue using α-thy-1 (a monoclonal antibody) and complement plus adherence. Enriched preparations of human Langerhans cells can be prepared by substituting an anti-CD 1 antibody for α-thy-1. In culture, neither mouse nor human Langerhans cells are active antigen-presenting cells until after 1-3 days in culture, after which time they enlarge, express more MHC Class II and cell adhesion molecules, and lose Fc receptors, fully resembling blood and lymphoid dendritic cells. Cell populations containing more than 90% dendritic cells have been obtained from human blood, where, without enrichment, fewer than 0.1% of the white cells are dendritic cells. Such enrichment can be achieved by successive depletion of T cells, monocytes, and B plus NK cells to yield an initial population ranging from 30-60% dendritic cells. Greater purity is then obtained by panning or FACS using a monoclonal antibody, especially to $CD45R_A$, that selectively reacts to contaminants (Freudenthal, P., Steinman, R.M., *Proc. Nat'l. Acad. Sci. (USA)* (1990) *87*:7698-7702.) To enrich for dendritic cells generally, selection for low buoyant density, non-adherence to plastic in culture (especially after one or more days), and absence of markers found on other cells is performed. Such methods deplete other cell types, but do not positively select dendritic cells.

Dendritic cells express a distinct pattern of markers on their cell membranes. Figure 1 illustrates this pattern by indicating the presence or absence of several distinct cell surface markers. Other markers which can be used to positively or negatively select for dendritic cells include ICAM-1 (CD 54), LFA-3 (CD 58), and CD 11b. Dendritic cells isolated from human or mouse blood, but not skin, express CD 11a or LFA-1. In skin, the immunostimulatory effect of dendritic cells may be enhanced by cytokines, particularly by GM-CSF.

Dendritic cells initiate T-dependent responses from quiescent lymphocytes. Once sensitized, T cells interact with other antigen presenting cells. Dendritic cell antigen processing activity is regulated. Only fresh cells, *i.e.*, cells cultured for less than a day, isolated form skin or lymphoid organs present native proteins. After that time, they do not process antigens. In addition, dendritic cells are not actively phagocytic.

Gene therapy-mediated immunostimulation can be accomplished by various methods. For example, an expression vector encoding a disease-associated antigen or modified form thereof (collectively referred to hereinafter as "antigen") can be delivered to dendritic cells to initiate an immune response against the antigen. Expression of the antigen may be transient or stable over time. Where an immune response is to be stimulated by an antigen from a pathogenic agent, *e.g.*, a virus, a bacteria, or neoplastic or otherwise diseased autologous cell, the expression vector preferably encodes a modified form of the antigen which has reduced pathogenicity relative to the native antigen but still stimulates an immune response thereto.

In the particular case of disease caused by viral infection (*e.g.*, AIDS caused by HIV), the immunity stimulating expression product encoded by the expression vector is of a form which will elicit either or both an HLA Class I- or Class II-restricted immune response. For HIV, a preferred antigen for immunostimulation is derived from the envelope protein, preferably selected from gp 160, gp 120, and gp 41, which has been modified to reduce pathogenicity, in particular, to reduce the possibility of syncytia, to avoid expression of epitopes leading to a disease enhancing immune response, to remove immunodominant but strain-specific epitopes, or to present several strain-specific epitopes. Other HIV genes or combinations of genes which may be expressed for this purpose include *gag, pol, rev, vif, nef, prot, gag/pol, gag prot, etc.* Additionally, immunogenic portions from other desired antigens may be expressed. Immunogenic portion(s) of desired antigens may be of varying length, preferably at least 9 amino acids and may include the entire protein. As those in the art will appreciate, this and similar immunostimulatory approaches can be employed in the treatment of numerous diseases.

In further embodiments, expression vectors direct expression of at least one gene of interest which encode one or more immunogenic portions of disease-associated antigens. As used herein, a gene of interest codes for at least one product capable of

immunostimulation. A "disease-associated" antigen is one associated with rendering a cell (or organism) diseased, or with the disease state in general but which is not required for rendering the cell diseased. A wide variety of "disease-associated" antigens are known, including immunogenic, non-tumorigenic altered cellular components which are normally associated with tumor cells (*see* WO 93/10814). Tumor associated antigens that correspond to a cell mediated immune response, such as MAGE-1, MAGE-3, MART-1, and gp100, are also included among "disease associated antigens" as used herein. "Disease-associated" antigens should also be understood to include all or portions of various eukaryotic, prokaryotic or viral pathogens. *See* WO 93/10814 for additional details.

Nucleic acid molecules that encode the above-described products, as well as other nucleic acid molecules that are advantageous for use within the present invention, may be readily obtained from a variety of sources, including, for example, depositories such as the American Type Culture Collection, or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Alternatively, cDNA sequences for use with the present invention may be obtained from cells which express or contain the sequences, such as by RT PCR from isolated mRNA. Nucleic acid molecules suitable for use with the present invention may also be synthesized in whole or in part, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, ABI DNA synthesizer model 392 (Foster City, CA).

A. Gene Delivery Vehicles

A gene delivery vehicle ("GDV") is a composition capable of delivering a nucleic acid molecule, specifically an expression vector, to a eukaryotic cell. Representative examples of gene delivery vehicles include recombinant viral vectors (*e.g.*, retroviruses; *see* WO 89/09271, and alphaviruses such as Sindbis; *see* WO 95/07994), other recombinant and non-recombinant viral systems (*e.g.*, adenovirus; *see* WO 93/19191), nucleic acid molecules associated with one or more condensing agents (*see* WO 93/03709), nucleic acid molecules associated with liposomes (Wang, *et al., PNAS* *84*:7851, 1987), modified bacteriophage, or bacteria. In whatever form, the GDV carries

an expression vector which directs expression of at least one disease associated antigen in a target cell.

In one embodiment, the GDV is a recombinant virus derived from a virus such as an astrovirus, coronavirus, orthomyxovirus, papovavirus, paramyxovirus, parvovirus, picornavirus, poxvirus, retrovirus, togavirus or adenovirus. In a preferred embodiment, the recombinant viral vector is a recombinant retroviral vector. Retroviral GDVs may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses, as well as spumaviruses and lentiviruses (*see* RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection (ATCC, Rockville, MD), or isolated from known sources using commonly available techniques. Numerous retroviral GDVs which may be utilized in practicing the present invention are described in U.S. Patent Nos. 5,219,740 and 4,777,127, EP 345,242 and WO 91/02805.

Particularly preferred recombinant retroviruses are derived from retroviruses which include avian leukosis virus (ATCC Nos. VR-535 and VR-247), bovine leukemia virus (VR-1315), murine leukemia virus (MLV), mink-cell focus-inducing virus (Koch *et al.*, *J. Vir. 49*:828, 1984; and Oliff *et al.*, *J. Vir. 48*:542, 1983), murine sarcoma virus (ATCC Nos. VR-844, 45010 and 45016), reticuloendotheliosis virus (ATCC Nos VR-994, VR-770 and 45011), rous sarcoma virus, Mason-Pfizer monkey virus, baboon endogenous virus, endogenous feline retrovirus (*e.g.*, RD114), gibbon ape leukemia virus (GALV), human immunodeficiency virus (HIV), HTLV I, HTLV III, and mouse or rat gL30 sequences used as a retroviral vector. Particularly preferred strains of MLV from which recombinant retroviruses can be generated include 4070A and 1504A (Hartley and Rowe, *J. Vir. 19*:19, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi (Ru *et al.*, *J. Vir. 67*:4722, 1993; and Yantchev *Neoplasma 26*:397, 1979), Gross (ATCC No. VR-590), Kirsten (Albino *et al.*, *J. Exp. Med. 164*:1710, 1986), Harvey sarcoma virus (Manly *et al.*, *J. Vir. 62*:3540, 1988; and Albino *et al.*, *J. Exp. Med. 164*:1710, 1986) and Rauscher (ATCC No. VR-998), and Moloney MLV (ATCC No. VR-190). A particularly preferred non-mouse retrovirus is rous sarcoma virus. Preferred rous sarcoma viruses include Bratislava (Manly *et al.*, *J. Vir. 62*:3540, 1988; and Albino *et al.*, *J. Exp. Med.*

164:1710, 1986), Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard (ATCC No. VR-140), Carr-Zilber (Adgighitov *et al.*, *Neoplasma 27*:159, 1980), Engelbreth-Holm (Laurent *et al.*, *Biochem Biophys Acta* 908:241, 1987), Harris, Prague (*e.g.*, ATCC Nos. VR-772, and 45033), and Schmidt-Ruppin (*e.g.* ATCC Nos. VR-724, VR-725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral GDVs given the disclosure provided herein and standard recombinant techniques. In addition, portions of the retroviral GDVs may be derived from different retroviruses. For example, recombinant retrovirus may comprises LTRs from a murine sarcoma virus, a tRNA binding site from a rous sarcoma virus, a packaging signal from a MLV, and an origin of second strand synthesis from an avian leukosis virus. These recombinant retroviral vectors may be used to generate transduction competent retroviral vector particles by introducing them into appropriate packaging cell lines (*see* WO 92/05266). In addition, recombinant retroviruses can be produced which direct the site-specific integration of the recombinant retroviral genome into specific regions of the host cell DNA. Such site-specific integration can be mediated by a chimeric integrase incorporated into the retroviral particle. *See, for example*, WO 96/06727. It is preferable that the recombinant viral vector is a replication defective recombinant virus.

Within another embodiment, GDVs are derived from togaviruses. Preferred togaviruses include alphaviruses, in particular, those described in co-owned WO 95/07994. A representative alphavirus in Sindbis virus. Briefly, recombinant Sindbis expression vectors typically comprise a 5' sequence capable of initiating Sindbis virus transcription, a nucleotide sequence encoding Sindbis non-structural proteins, a modified or inactivated viral junction region, a Sindbis RNA polymerase recognition sequence, at least one gene of interest, and a polyadenylate tract. Corresponding regions from other alphaviruses may be used in place of those described above.

In a preferred embodiment, a recombinant alphaviral vector does not encode structural proteins and the gene(s) of interest are located downstream from the viral junction region. In vectors having a second viral junction region, the gene(s) of interest may be located downstream from the second viral junction region. In such instances, the

vector may further comprise a polylinker located between the viral junction region and the gene(s) of interest.

Other recombinant togaviral vectors that may be utilized in the present invention include those derived from Semliki Forest virus (ATCC VR-67; ATCC VR-1247), Middleberg virus (ATCC VR-370), Ross River virus (ATCC VR-373; ATCC VR-1246), Venezuelan equine encephalitis virus (ATCC VR-923; ATCC VR-1250; ATCC VR-1249; ATCC VR-532), and those described within U.S. Patent Nos. 5,091,309, 5,217,879, and WO 92/10578. The above described recombinant Sindbis expression vector, as well as numerous similar vector constructs, may be readily prepared essentially as described in WO 95/07994.

In another embodiment, the recombinant viral vector is a recombinant adenoviral vector. Such expression vectors may be readily prepared and utilized given the disclosure provided herein (*see* Berkner, *Biotechniques 6*:616, 1988, and Rosenfeld *et al.*, *Science 252*:431, 1991, WO 93/07283, WO 93/06223, and WO 93/07282).

Other viral vectors suitable for use in the present invention include, for example, those derived from poliovirus (Evans *et al.*, *Nature 339*:385, 1989, and Sabin *et al.*, *J. Biol. Standardization 1*:115, 1973) (ATCC VR-58); rhinovirus (Arnold *et al.*, *J. Cell. Biochem.* L401, 1990) (ATCC VR-1110); pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch *et al.*, *PNAS 86*:317, 1989; Flexner *et al.*, *Ann. N.Y. Acad. Sci. 569*:86, 1989; Flexner *et al.*, *Vaccine 8*:17, 1990; U.S. 4,603,112 and U.S. 4,769,330; WO 89/01973) (ATCC VR-111; ATCC VR-2010); SV40 (Mulligan *et al.*, *Nature 277:*108, 1979) (ATCC VR-305), (Madzak *et al.*, *J. Gen. Vir. 73*:1533, 1992); influenza virus (Luytjes *et al.*, *Cell 59*:1107, 1989; McMicheal *et al.*, *The New England Journal of Medicine 309*:13, 1983; and Yap *et al.*, *Nature 273*:238, 1978) (ATCC VR-797); parvovirus such as adeno-associated virus (Samulski *et al.*, *J. Vir. 63*:3822, 1989, and Mendelson *et al.*, *Virology 166*:154, 1988) (ATCC VR-645); herpes simplex virus (Kit *et al.*, *Adv. Exp. Med. Biol. 215*:219, 1989) (ATCC VR-977; ATCC VR-260); *Nature 277*: 108, 1979); human immunodeficiency virus (EPO 386,882, Buchschacher *et al.*, *J. Vir. 66*:2731, 1992); measles virus (EPO 440,219) (ATCC VR-24); A (ATCC VR-67; ATCC VR-1247), Aura (ATCC VR-368), Bebaru virus (ATCC VR-600; ATCC VR-1240), Cabassou (ATCC VR-922), Chikungunya virus (ATCC VR-64; ATCC VR-1241), Fort

Morgan (ATCC VR-924), Getah virus (ATCC VR-369; ATCC VR-1243), Kyzylagach (ATCC VR-927), Mayaro (ATCC VR-66), Mucambo virus (ATCC VR-580; ATCC VR-1244), Ndumu (ATCC VR-371), Pixuna virus (ATCC VR-372; ATCC VR-1245), Tonate (ATCC VR-925), Triniti (ATCC VR-469), Una (ATCC VR-374), Whataroa (ATCC VR-926), Y-62-33 (ATCC VR-375), O'Nyong virus, Eastern encephalitis virus (ATCC VR-65; ATCC VR-1242), Western encephalitis virus (ATCC VR-70; ATCC VR-1251; ATCC VR-622; ATCC VR-1252), and coronavirus (Hamre *et al.*, *Proc. Soc. Exp. Biol. Med.* *121*:190, 1966) (ATCC VR-740).

In another embodiment of the invention, the GDV comprises a DNA or RNA expression vector. Representative examples of such GDVs include plasmids, cosmids, and linear double and single stranded polynucleotides. Such vectors should include the genetic information required for expression of the disease associated antigen(s) in dendritic cells, *i.e.*, a promoter, an open reading frame encoding the disease associated antigen, a transcription termination signal, and a polyadenylation signal. One advantage of such polynucleotide systems is the ease with which large quantities of a given gene delivery vehicle can be produced. Large amounts of RNA may be produced by *in vitro* transcription using any of a variety of techniques known in the art. Similarly, large amounts of DNA can readily be cultured in bacteria.

In a related embodiment, the GDV comprises a DNA or RNA expression vector associated with a condensing agent (*e.g.*, polycations). Polycations condense the expression vector by masking the negatively charged phosphate backbone, permitting the molecule to fold into a more compact form. Polycations useful in this embodiment of the invention include, among others, polylysine, polyarginine, histones, protamines, spermidine, spermine, and other highly basic proteins or polypeptides. Such polycations may be modified to incorporate targeting elements and/or to decrease immunogenicity of nucleic acid/polycation complexes. For example, the polycation can be chemically conjugated to one or more polyalkylene glycols, *e.g.*, polyethylene glycol. Alternatively (or additionally), the polycation can be conjugated with one or more polysaccharides. Briefly, chemical conjugation involves forming a covalent linkage between the polycation and the polyalkylene or polysaccharide. Many suitable methods for making such linkages are known in the art, for instance, *see* co-owned WO 96/21036.

In an alternative embodiment, the GDV is a DNA or RNA expression vector associated with a liposome. Liposomes are small, lipid vesicles comprised of an aqueous compartment enclosed by a lipid bilayer, typically spherical or slightly elongated structures several hundred Angstroms in diameter. Under appropriate conditions, a liposome can fuse with the plasma membrane of a cell or with the membrane of an endocytic vesicle within a cell which has internalized the liposome to release its contents into the cytoplasm. Prior to interaction with the surface of a cell, however, the liposome membrane acts as a relatively impermeable barrier which sequesters and protects its contents, for example, from degradative enzymes in the plasma. Additionally, because a liposome is a synthetic structure, specially designed liposomes can be produced that incorporate desirable features (*see* Stryer, L., *Biochemistry*, pp:236-240, 1975 (W.H. Freeman, San Francisco, CA); Szoka *et al.*, *Biochim. Biophys. Acta 600*:1, 1980; Bayer *et al.*, *Biochim. Biophys. Acta. 550*:464, 1979; Rivnay *et al.*, *Meth. Enzymol. 149*:119, 1987; Wang *et al.*, *PNAS 84*: 7851, 1987, Plant *et al.*, *Anal. Biochem. 176*:420, 1989, and U.S. Patent No. 4,762,915). Liposomes can encapsulate a variety of nucleic acid molecules including DNA, RNA, plasmids, and other expression vectors useful in the practice of the present invention.

GDVs, including GDVs coupled to one or more distinct dendritic cell targeting element types, can be further modified. One particularly useful modification includes use of compounds conjugated to polycations and gene delivery vehicles that reduce immunogenicity of the resulting compositions. *See* co-owned WO 96/21036.

B. Genes of Interest

GDVs useful in the practice of this invention above include or contain one or more genes of interest, *i.e.*, nucleic acid molecules which encode at least one antigen associated with or characteristic of the disease to be treated or prevented. Such antigens include those from altered cellular components and antigens from foreign organisms or other pathogens. A variety of other disease-associated antigens may be used in the gene delivery vehicles of the invention. See e.g., WO 91/02805, WO 96/20414 and U.S. Patent No. 5,580,859.

In other embodiments of the invention, expression vectors are provided which direct expression of immunogenic portions of antigens from foreign organisms or other

pathogens. These antigens are also referred to herein as "disease associated antigens." Representative examples of such antigens include antigens from bacteria (*e.g.*, *E. coli*, streptococcus, staphylococcus, mycobacteria, *etc.*), fungi, parasites, and viruses (*e.g.*, influenza virus, HIV, and Hepatitis A, B and C Virus ("HAV", "HBV" and "HCV", respectively), human papiloma virus ("HPV"), Epstein-Barr Virus ("EBV"), herpes simplex virus ("HSV"), hantavirus, HTLV I, HTLV II, cytomegalovirus ("CMV"), and feline leukemia virus). As utilized herein, "immunogenic portion" refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (*i.e.*, cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 9 amino acids long, and may include the entire antigen.

Within one embodiment of the invention, expression vectors encode, in addition to at least one disease associated antigen, a gene encoding an immunomodulatory cofactor, *i.e.*, a factor which, when expressed in dendritic cells in addition to the disease associated antigen(s) causes the immune response to the antigen(s) to be enhanced in quality or potency from that which would have occurred in the absence of the cofactor. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, *in vitro* assays which measure cellular proliferation (*e.g.*, ^{3}H thymidine uptake), and *in vitro* cytotoxic assays (*e.g.*, which measure ^{51}Cr release) (*see* Warner et al., *AIDS Res. and Human Retroviruses* 7:645-655, 1991). In alternative embodiments, an immunomodulatory cofactor is, instead of be encoded by the expression vector, added exogenously either before, simultaneous with, or after the gene delivery vehicle is administered.

Immunomodulatory factors may be active both *in vivo* and *ex vivo*. Representative examples of such immunomodulatory factors include, for example, cytokines, such as IL-1, IL-2 (Karupiah *et al.*, *J. Immunology 144*:290, 1990; Weber *et al.*, *J. Exp. Med. 166*:1716, 1987; Gansbacher *et al.*, *J. Exp. Med. 172*:1217, 1990; U.S. Patent No. 4,738,927), IL-3, IL-4 (Tepper *et al.*, *Cell 57*:503, 1989, Golumbek *et al.*, *Science 254*:713, 1991 and U.S. Patent No. 5,017,691), IL-5, IL-6 (Brakenhof *et al.*, *J. Immunol. 139*:4116, 1987, and WO 90/06370), IL-7 (U.S. Patent No. 4,965,195) , IL-8, IL-9, IL-10, IL-11, IL-12 (Wolf *et al.*, *J. Immuno. 46*:3074, 1991 and Gubler *et al.*, *PNAS 88*:4143, 1991), IL-13 (WO 94/04680), IL-14, IL-15, α-interferon (Finter *et al.*, *Drugs 42*(5):749,

1991, Nagata *et al.*, *Nature* 284:316, 1980; Familletti *et al.*, *Methods in Enz. 78*:387, 1981, Twu *et al.*, *PNAS USA 86*:2046, 1989, Faktor *et al.*, *Oncogene 5*:867, 1990, U.S. Patent No. 4,892,743, U.S. Patent No. 4,966,843, and WO 85/02862), β-interferon (Seif *et al.*, *J. Vir. 65*:664, 1991), γ-interferons (Radford *et al.*, *The American Society of Hepatology* 9:2008, 1991, Watanabe *et al.*, *PNAS 86*:9456, 1989, Gansbacher *et al.*, *Cancer Research 50*:7820, 1990, Maio *et al.*, *Can. Immunol. Immunother. 30*:34, 1989, U.S. Patent No. 4,762,791, and U.S. Patent No. 4,727,138), G-CSF (U.S. Patent Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), tumor necrosis factors (TNFs) (Jayaraman *et al.*, *J. Immunology 144*:942, 1990), CD3 (Krissanen *et al.*, *Immunogenetics 26*:258, 1987), CD8, ICAM-1 (Altman *et al.*, *Nature 338*:512, 1989; Simmons *et al.*, *Nature 331*:624, 1988), ICAM-2 (Singer *Science 255*:1671, 1992), LFA-1 (Altmann *et al.*, *Nature 338*:521, 1989), LFA-3 (Wallner *et al.*, *J. Exp. Med. 166*(4):923, 1987), and other proteins such as HLA Class I molecules, HLA Class II molecules, B7 (Freeman *et al.*, *J. Immuno. 143*:2714, 1989), B7-2, β_2-microglobulin (Parnes *et al.*, *PNAS 78*:2253, 1981), chaperones, and MHC linked transporter proteins or analogs thereof (Powis *et al.*, *Nature 354*:528, 1991). The choice of which immunomodulatory factor(s) to employ is based upon the therapeutic effects of the factor. Preferred immunomodulatory factors include α-interferon, γ-interferon, and IL-2.

Nucleic acid molecules encoding disease associated antigens can be readily obtained from a variety of sources, including for example, depositories such as the American Type Culture Collection (ATCC, Rockville, MD), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford, England). Alternatively, cDNAs which encode such antigens may be generated from cells known to express or contain the corresponding gene (*see* U.S. Patent Nos. 4,683,202; 4,683,195 and 4,800,159. *See also PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989). Alternatively, genes of known sequence, or which encode antigens of known amino acid sequence, may be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, APB DNA synthesizer model 392 (Foster City, CA)).

C. TARGETING ELEMENTS

As discussed above, one aspect of the present invention provides compositions and methods for targeting a gene delivery vehicle to a dendritic cell, either *in vivo* or *in vitro*. In the context of this invention, a targeting element is a molecule that has affinity for a molecule present on the surface of a dendritic cell. A wide variety of targeting elements can be utilized in the practice of this invention to specifically direct a gene delivery vehicle to a dendritic cell.

One embodiment of the invention concerns the use of a high affinity bind pair to target a gene delivery vehicle to a dendritic cell. In other embodiments where targeting elements are employed, the targeting element is covalently linked to a gene delivery vehicle via a multifunctional linking agent. *See generally* co-owned WO 92/05266.

As will be appreciated by those skilled in the art, other targeting mechanisms which enable dendritic cell targeting also fall within the scope of the invention.

D. GDV Production

Once the GDV has been designed, it must be produced in an amount sufficient for conjugation to a desired targeting element and/or for administration to an animal. If the GDV is a recombinant viral vector, it may be produced utilizing a packaging system. A variety of viral vector packaging systems are described below in which one or more essential functions of the parent virus has been deleted so that it is deficient in some function (*e.g.*, genome replication), but retains a packaging signal and the ability to express gene products from one or more nucleic acid molecules. Representative examples of viral vector packaging systems include those for retroviral vectors, alphaviral vectors and adenoviral vectors.

When such recombinant retroviral vectors are utilized, it is preferable to utilize packaging cell lines for producing viral particles wherein at least the codons of 5' terminal end of the *gag/pol* gene are modified to take advantage of the degenerate nature of the genetic code to minimize the possibility of homologous recombination between the vector and sequences in the packaging cell coding for the viral structural proteins. Additional techniques for reducing the possibility of recombination events between vectors present in

a packaging cell and the recombinant retroviral genome to be packaged are provided in the co-owned WO 95/30763 and WO 96/07749.

Packaging cell lines suitable for use with the above described recombinant retroviral vectors may be readily prepared using techniques known in the art (*see* WO 95/30763 and WO 92/05266), and utilized to create producer cell lines for the production of recombinant vector particles.

In a further embodiment of the invention, alphavirus packaging cell lines are provided. In particular, alphavirus packaging cell lines are provided wherein the viral structural proteins, supplied in *trans* from one or more stably integrated expression vectors, are able to encapsidate transfected, transduced, or intracellularly produced vector RNA transcripts in the cytoplasm and release infectious, packaged vector particles through the cell membrane, thus creating an alphavirus vector producing cell line.

Packaging cell lines suitable for use with the above described alphaviral vector constructs may be readily prepared (*see* WO 95/07994).

Within further embodiments of the invention, adenovirus packaging cell lines are provided. Adenovirus vectors are derived from nuclear replicating viruses and may be constructed such that they are replication defective. One or more nucleic acid molecules may be carried by adenoviral vectors for delivery to target cells (*see* Ballay *et al.*, *EMBO J. 4*:3861, 1985, Thummel *et al., J. Mol. Appl. Genetics 1*:435, 1982 and WO 92/05266).

Within another embodiment of the invention, a targeted gene delivery vehicle may include one or more fusigenic proteins to assist in gene delivery. Representative fusigenic proteins include ecotropic murine retrovirus envelope proteins, other retrovirus envelope proteins modified to disable normal receptor recognition, fusigenic proteins from herpes simplex virus fusigenic proteins gH and gL, Epstein-Barr virus fusigenic proteins, measles virus proteins, malarial sporozoite fusigenic proteins, and other proteins known in the art to have fusogenic properties.

E. Purification of Gene Delivery Vehicles

Once the GDVs are produced, they are preferably purified prior to conjugation to the desired targeting element. In addition, compositions comprising targeted GDVs are

preferably purified again prior to administration. The techniques utilized for purification is dependent on the type of GDV to be purified. For example, there are a variety of techniques known in the art which may be used if the GDV is an enveloped recombinant viral vector, a nucleic acid or a liposome. (*See* U.S. Patent No. 5,447,859 for an example of purification procedures.)

In addition, if the GDV is a nucleic acid, there are a variety of techniques known in the art including, for example, purification by CsCl-ethidium bromide gradient, ion-exchange chromatography, gel-filtration chromatography, and differential precipitation with polyethylene glycol. Further description of the purification of nucleic acids is provided in Sambrook *et. al.*, *Molecular Cloning: A Laboratory Manual*, 2d ed. (Cold Spring Harbor Laboratory Press, 1989).

When the GDV is a liposome, a variety of purification methods known to those skilled in the art may be utilized and are described in more detail in Mannino and Gould-Fogerite (*BioTechniques 6*:682, 1988). Briefly, preparation of liposomes typically involves admixing solutions of one or more purified phospholipids and cholesterol in organic solvents and evaporating the solvents to dryness. An aqueous buffer containing the GDVs is then added to the lipid film and the mixture is sonicated to create a fairly uniform dispersion of liposomes. In certain embodiments, dialysis, gel filtration, or ultracentrifugation is then be used to separate unincorporated components from the intact liposomes. (Stryer, L., *Biochemistry*, pp:236 1975 (W.H. Freeman, San Francisco); Szoka *et al.*, *Biochim. Biophys. Acta 600*:1, 1980; Bayer *et al.*, *Biochim. Biophys. Acta. 550*:464, 1979; Rivnay *et al.*, *Meth. Enzymol. 149*:119, 1987; Wang *et al.*, *PNAS 84*: 7851, 1987 and, Plant *et al.*, *Anal. Biochem. 176*:420, 1989.

F. Formulation

Following purification of a composition comprising a GDV, the preparation is preferably formulated into a pharmaceutical composition. Such compositions may be in liquid or dry form. Preferred liquid compositions comprise aqueous solutions in which the desired product is suspended in a pharmaceutically acceptable carriers and/or diluent and may additionally comprise stabilizers, and other excipients. Alternatively, the compositions of the invention can be prepared in solid formulations intended for

resuspension just prior to use. Dry formulations include those which are lyophilized or freeze dried.

When GDV is a lipid enveloped virus, preferred dry formulations comprise some or all of the following: one or more pharmaceutically acceptable carriers and/or diluents; a saccharide; a high molecular weight structural additive; a buffering component; water; and one or more amino acids. The combination of some or all of these components acts to preserve the activity of the targeted GDV upon freezing and lyophilization, or drying through evaporation. Pharmaceutically acceptable carriers or diluents according to the invention are non-toxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include for example water, isotonic saline solutions (*i.e.,* phosphate-buffered saline or Tris-buffered saline, preferably buffered at physiological pH), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin.

A preferred lyophilized composition for lipid enveloped viruses, particularly recombinant retroviruses and alphaviruses, comprises 10 mg/mL mannitol, 1 mg/mL HSA, 20 mM Tris, pH 7.2, and 150 mM NaCl being particularly preferred (*see* WO 95/10601). Such compositions are stable at -70°C for at least six months. The pharmaceutical compositions of the invention may also additionally include factors to stimulate cell division, and hence, uptake and incorporation of the administered GDVs. Particularly preferred methods and compositions for preserving recombinant viruses are described in WO 95/10601, WO 95/07994 and WO 96/21014). In liquid or solid form, after preparation of the composition where the GDV is a recombinant virus, the recombinant virus preferably will constitute about 10 ng to 1 mg of material per dose, with about 10 times this amount of material present as copurified contaminants. Preferably, the composition is administered in doses of about 0.1 to 1.0 mL of aqueous solution, which may or may not contain one or more additional pharmaceutically acceptable excipients, stabilizers, or diluents.

I. Administration

Compositions according to the invention, be they dendritic cells modified *ex vivo* or GVDs for direct administration, are typically administered *in vivo* via parenteral (*e.g.*,

intravenous, subcutaneous, and intramuscular) or other traditional direct routes, such as buccal/sublingual, rectal, oral, nasal, topical, (such as transdermal and ophthalmic), vaginal, pulmonary, intraarterial, intraperitoneal, intraocular, or intranasal routes or directly into a specific tissue, such as the liver, bone marrow, or into the tumor in the case of cancer therapy. Non-parenteral routes are discussed further in WO 96/20732.

Preferably, the composition is administered to an animal via the desired route and then the animal is tested for the desired biological response. Such testing may include immunological screening assays *e.g.*, CTL assays, antibody assays. The test(s) performed will depend on the product produced by the nucleic acid molecule introduced by the targeted GDV and the disease to be treated or prevented. On the basis of the results of such testing, the titers of the targeted GDVs to be administered may be adjusted to further enhance the desired effect(s) if more than administration is required.

Administration by many of the routes of administration described herein or otherwise known in the art may be accomplished simply by direct administration using a needle, catheter or related device, at a single time point or at multiple time points. In addition, an "administration" of a gene delivery vehicle (or ex vivo transduced cells, for that matter) at a given time point includes administration to one or more areas, or by one or more routes. In certain embodiments of the invention, one or more dosages is administered directly in the indicated manner: intravenously at dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu; intraarterially at dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu; intramuscularly at dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu, with dosages of 10^{10} or 10^{11} cfu being preferred; intradermally at dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu; pulmonarily at dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu; subcutaneously at dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu, with dosages of 10^9, 10^{10} or 10^{11} cfu being preferred; interstitially at dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu, with dosages of 10^8, 10^9, 10^{10} or 10^{11} cfu being preferred; into a lymphoid organ such as the spleen, a tonsil, or a lymph node at

dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu; into a tumor at dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu, with dosages of 10^8, 10^9, 10^{10} or 10^{11} cfu being preferred; intraperitoneally at dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu, with dosages of 10^8, 10^9, 10^{10} or 10^{11} cfu being preferred; and into the afferent lymph at dosages greater than or equal to 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} or 10^{11} cfu. For purposes of the convenience, "cfu" shall also refer to non-viral particles, such that one cfu is equivalent to one non-viral particle.

Other routes and methods for administration include non-parenteral routes, such as are disclosed in co-owned WO 96/20732, as well as administration via multiple sites, as disclosed in co-owned WO 96/20731.

Examples

The following examples are included to more fully illustrate the present invention. Additionally, these examples provide preferred embodiments of the invention and are not meant to limit the scope thereof. Standard methods for many of the procedures described in the following examples, or suitable alternative procedures, are provided in widely reorganized manuals of molecular biology, such as, for example "Molecular Cloning," Second Edition (Sambrook, *et al.*, Cold Spring Harbor Laboratory Press, 1987) and "Current Protocols in Molecular Biology" (Ausubel, *et al.*, eds. Greene Associates/Wiley Interscience, NY, 1990).

Example 1

Identification of Dendritic Cells as Key Antigen Presenting Cells Following Direct Administration of Gene Delivery Vehicles

As discussed above, direct injection of recombinant retroviruses carrying an expression vector coding for at least one disease associated antigen vector is preferred to *ex-vivo* approaches. However, compared to an *ex vivo* approach where the desired disease associated antigen is expressed by transduced cells, *e.g.*, fibroblasts, which are then administered to a patient, direct administration of gene delivery vehicles to a patient lead

to transduction of cells at and adjacent to the injection site, as well as transducing cells residing in tissues where the gene delivery vehicle can be transported, such as to lymphoid tissue following intravenous or subcutaneous administration. Previously, it was unknown which cells were responsible for presenting the disease associated antigen(s) encoded by the expression vector directly delivered by injection of gene delivery vehicles. Identification of transduced cells which present antigen(s) encoded by an expression vector delivered by direct injection to an animal is described below.

In previous biolocalization studies designed to trace expression vectors delivered by a recombinant retrovirus directly injected into animals, whole organ PCR was used to detect proviral DNA at the site of injection (Sajjadi, *et al.*, 1994; Kamantigue, *et al.*, 1995). In addition, the proviral DNA was sometimes detected at secondary lymphoid organs, a result consistent with the observation that these animals mounted excellent immune responses to the immunogen encoded by the provirus.

In this study, four expression vectors delivered using recombinant retroviruses, each encoding either HIV gp160/120 envelope, bacterial β-galactosidase, chicken ovalbumin, or luciferase, were used to identify cells involved in antigen presentation and immune induction following direct administration. Four cell populations were examined. The first cell population contains proviral copies of the expression vector (Group I), as determined PCR. The second subset of cells are those which express the encoded protein (Group II). β-gal histochemistry and immunohistochemistry assays were employed to analyze these cells. The third subset (Group III) consists of subpopulations of Group II cells that can present antigenic peptides to the immune system. Group III cells were identified by their ability to induce immune responses in T cell assays. Finally, Group IV consists of a distinct group of cells which do not contain proviral copies of the expression vector and thus do not express the antigenic protein or peptide from its corresponding gene, but nonetheless present the antigen(s). Examples of Group IV cells include phagocytic cells which engulf primary transduced cells or their fragments. Such cells then reprocess and present antigenic peptides in the context of their own MHC molecules.

Retroviral Vectors

Non-replicating, amphotropic murine recombinant retroviruses carrying expression vectors encoding HIV gp160/120, bacterial β-galactosidase, luciferase and chicken ovalbumin were used. Retroviral vector backbones and high titer viral preparations (>1 x10^7 cfu/mL) were generated as described in WO 89/09271, WO 92/05266, and co-owned WO 96/21014. A 1.2 kb Bam HI to Bgl II fragment encoding a cDNA of chicken ovalbumin in pUG-1 vector (Moore, *et al.*, 1993) was cloned into the Bam HI site of pSP73 (Promega, Madison WI). A 1.2 kb Xho I to Cla I fragment was recovered and substituted for the corresponding 3.8 kb Xho I to Cla I fragment from N2-HIV gp160/120. Aliquots of the viral preparations were tested for replication-competent retrovirus (RCR) and determined to be RCR-free.

Animals and Vector Immunizations

Six- to eight- weeks old female BALB/c (H-2^d) or C57BL/6 (H-2^b) mice from Harlan Sprague-Dawley were used in all experiments. On days 1, 4 and 7, mice were injected either intramuscularly (i.m.) in both gastrocnemius or Tibialis anterior muscles with 100 µl/injection, for a total of 200 µl/mouse or intradermally at the base of tail with 100 µl of the retroviral preparations. Spleens were harvested and *in vitro* CTL cultures were initiated by mixing with appropriate irradiated splenocytes.

Immunohistochemistry

Antibodies to the following lymphocyte markers were used as culture supernatants or purified immunoglubulins: CD4 (rat IgG); CD8 (rat IgM); Macrophages (rat IgG); and B220 (rat (IgM). Commercially available biotinylated antibodies to CD45 (30R11.1, rat IgG; Pharmingen, San Diego, CA), rat Ig (polyclonal; Jackson ImmunoResearch Labs., Inc., West Grove, PA), and rabbit Ig (polyclonal; Jackson ImmunoResearch, PA) were also used. Normal rabbit serum was used as a negative control.

Muscles from injection sites were carefully excised, immersed in OCT and rapidly frozen in liquid nitrogen and stored at -70°C until use. Cryostat sections at 5-6 µm were cut, air dried and fixed in acetone for 5 min. Sections were incubated with previously titrated optimal concentrations of antibodies diluted in Tris-buffered saline (pH 7.4) for 60

min., washed in Tris-buffered saline, incubated with biotinylated anti-rat or rabbit IgG, and washed and then incubated with streptavidin conjugated alkaline phosphatase (Jackson ImmunoResearch, PA). Bound alkaline phosphatase was detected with the substrate Fast-violet, as previously described (Surh, *J. Exp. Med., vol.* 176:495-505). The sections were lightly counterstained with Meyer's hematoxylin and photographed under a Zeiss Axioscope microscope. To minimize background, antibodies were used after spinning in a Airfuge ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) at 148,000 x g for 30 min. and discarding the pellet.

Isolation of Infiltrating Cells

Injection sites were visualized via fluorescent emission from co-injected latex beads followed by excision and dicing with surgical scissors. Resulting small pieces of muscle (average sizes of 0.5 cm^3) were digested with trypsin at 37°C for 1 hr. with occasional shaking. At the end of the digestions, fetal calf serum was added and residual muscle pieces were separated by gravity. Infiltrating cells were washed with media and counted followed by luciferase and B3.Z assays.

Splenocyte Fractionation by Percoll Density Gradients (see FIG. 2)

Percoll stock was prepared by mixing 9 parts Percoll to 1 part 10X phosphate buffered saline (PBS). "1.030" and "1.075" working Percoll solutions were made by mixing 75% PBS plus 25% Percoll stock solutions and 40% PBS plus 60% Percoll stock solutions, respectively. Splenocytes from naive or immunized animals were fractionated into B plus T cells, macrophages and dendritic cells by following methods. Spleens were mashed in DNAse (Calbiochem, 3800 units/mL) in Hank's balanced salt solutions (HBSS) at room temperature with occasional rocking for 1 hour, followed by filtering through nylon mesh to remove debris. Resulting cells were pooled and centrifuged at 1500 rpm for 5 min., followed by resuspension in "1.075" Percoll solutions. "1.075" Percoll solutions containing the pooled splenocytes were carefully overlayed with "1.030" Percoll solutions and the gradients were spun for 20 min. at 1200 rpm. After the Percoll centrifugation, low density cells in the interface and high density cells in the pellet were separately recovered and washed extensively. High density cells included red blood cells, T and most of the B

cells except plasma cells. Low density cells included some B cells (including plasma cells), dendritic cells, monocytes and macrophages, but no T cells as judged by FACS. Most of the B cells in low density fractions were removed by careful washing after incubating cells for 2 hr. in tissue culture plates with complete medium (RPMI1640, 10% fetal bovine serum). Remaining adherent cells, which included dendritic cells, monocytes and macrophages, were further incubated overnight. Mature macrophages were separated from all other cell types since they are the only cells which still adhere after overnight culture.

X-gal (5-bromo-4-chloro-3-indosyl b-D-galactopyranoside) Staining

Cultures were fixed with 3% formaldehyde for 5 min. at room temperature, followed by washing with PBS. The cells were overlayed with a solution of 1 mg/mL X-gal/mL, 5 mM potassium ferrocyanide, 5 mM of potassium ferricyanide, and 2 mM $MgCl_2$. The plates were examined microscopically for the presence of LacZ expressing, blue cells after an overnight incubation at 37°C.

In vitro CTL Induction and Cytotoxicity Assays

Five mice were immunized by administering recombinant retroviruses carrying expression vectors encoding HIV gp160/120. B + T cells, dendritic cells and macrophage fractions were prepared. Five non-immunized litter mates were processed in an analogous manner. Cell fractions were mixed with irradiated stimulators at a 50:1 ratio and cultured for 7 days. ^{51}Cr release assays (Warner, *et al.*(1991),*AIDS Res. and Human Retroviruses, vol.* 7:645-655) were performed and percent lysis was calculated.

Ex vivo Transduction of Dendritic Cells and Monocytes and Adoptive Transfer

Naive mice were sacrificed and low density fractions were prepared as described above and put into in media containing GM-CSF (25 units/mL) and IL-4 (100 units/mL). DA/KT-1 and DA/β-gal producer cell lines were seeded in transwells with pore sizes of 0.45 micron. Dendritic cells and producer cells in transwells were co-cultured for three days. Recovered cells were extensively washed. Naive recipient mice were injected intraperitoneally with these transduced dendritic cells or fibroblasts stably transduced with

HIV gp160/120. Spleens from recipient mice were harvested 7 days later, followed by *in vitro* restimulation and ^{51}Cr release assays.

B3.Z assays

B3.Z cells are T cell hybridomas which recognize chicken ovalbumin in the context of H-2K^b molecules. They contain a plasmid containing the bacterial LacZ gene fused to the minimal promoter of the human IL-2 gene, and are therefore LacZ inducible upon antigenic stimulation. Antigen presenting cells are either cells transfected with ovalbumin gene or fractionated splenocytes from ovalbumin expression vector immunized mice. Individual cultures containing 2 x 10^5 B3.Z cells and 1 x 10^7 antigen presenting cells were incubated overnight, followed by X-gal staining to assess the level of B3.Z activation. Numbers of antigen presenting cells in each fraction were estimated from the standard curve generated from cultures containing known numbers of cells transfected with ovalbumin genes.

Results

To define the cell types which are transduced and induce immunity following direct administration to muscle of recombinant retrovirus-based gene delivery vehicles, injection sites were examined by immunohistochemisty to identify *in vivo* transduced cells. Mice were immunized with a recombinant retrovirus carrying an expression vector encoding chicken ovalbumin. After three injections, mice were sacrificed for cryosection and the injection sites were analyzed by immunohistochemistry with antibodies against a variety of leukocyte markers and ovalbumin.

Many infiltrating cells were found in the endomesium near the injection sites. From the marker profile analyses, F4/80 positive monocytes/macrophages were identified as the most prominent infiltrating cells at the injection sites. Additionally, a significant number of CD4 positive cells and occasional B cells infiltrated at the injection sites. No CD8 positive cells were found. Two dendritic cell-specific antibodies against distinct epitopes, NLDC-145 and N418, enabled detection of significant numbers of dendritic cells at the injection sites. Mice injected with formulation buffer only showed comparable

lymphocyte infiltration, suggesting the needle injury from injection is sufficient enough to cause lymphocyte infiltration. When the intradermal route of injection (which induces comparable immune responses) was examined, injection sites showed comparable infiltration by cells with the same phenotypes.

By immunohistochemistry, some infiltrating cells were found to express ovalbumin proteins. Double staining with anti-ovalbumin and F4/80 antibodies indicated that some, but not all, ovalbumin positive cells were co-stained with the macrophage marker, F4/80. To verify injection site expression of proteins encoded by the expression vectors, infiltrating cells were isolated from animals injected with luciferase- or ovalbumin-encoding gene delivery vehicles. Cells were first analyzed by NBT assays, verifying that most of them are indeed of the macrophage/monocyte lineage. Next, infiltrating cells were subjected to either luciferase or B3.Z assays. Luciferase assays revealed that most of the luciferase activity was concentrated in infiltrating cells, but not in residual muscles. Furthermore, B3.Z assays indicate that the infiltrating cells present ovalbumin-derived peptides to T cells. No staining was found using anti-ovalbumin antibodies in parallel sections with normal rabbit serum or at the injection sites in mice treated with HIV gp160/120-encoding gene delivery vehicles. These results indicate that some fraction of infiltrating cells at the injection sites were indeed transduced and express antigenic proteins which can be processed to stimulate immune responses.

From these experiments, inflammation caused by needle injury likely promotes the infiltration of leukocytes which then become transduced by gene delivery vehicles. These transduced leukocytes then migrate to secondary lymphoid organs to elicit immune responses. To confirm this, proviral DNA integration and protein expression was analyzed in various leukocyte populations in lymph nodes and spleens.

Previous biolocalization studies using DNA isolated from whole organs led to detection of proviral DNA in some secondary lymphoid organs (Sajjadi, *et al.*, *supra*; Kamantigue *et al.*, *supra*). Because of the limited sensitivity of whole organ PCR, PCR assays were performed on fractionated spleen cells. Splenocytes from immunized mice were pooled and separated into B cells, T cells, macrophages and dendritic cell fractions. In mice immunized with genes coding for HIV gp160/120 or β-gal, in addition to a marker

gene conferring resistance to neomycin, the neo markers encoded by both vectors were detected in the dendritic and macrophage cell fractions.

Since proviral DNA integration was detected in leukocyte cell fractions, expression vector-derived protein expression was also analyzed in these transduced cell fractions. BALB/c mice were injected three times with gene delivery vehicles directing β-gal expression. Their spleens were pooled and fractionated as described above, followed by X-gal staining. In agreement with PCR data, unfractionated spleen and dendritic cell fractions showed β-gal staining. Most of the transduced cells were found in clusters, suggesting they were clonally derived from the originally transduced cells. Here, 0.2% of dendritic cell fractions were transduced, corresponding to about 2,000 cells per mouse. In addition, macrophage fractions and cells from inguinal and popiloteal lymph nodes of these mice showed some X-gal staining, although the numbers were greatly reduced compared to the dendritic cell fractions. The high density fractions, containing B and T cells, were negative for X-gal staining.

Phenotypes of the transduced cells were further analyzed. B cells, T cells and macrophage fractions were shown to be homogenous following fractionation, as judged by their representative marker profiles for CD45R/B220, Thy-1.2. and F4/80. In contrast, cells adhering to plastic immediately after the cell isolation but not after overnight culturing contained mostly dendritic cells based on several dendritic cell antibody stainings, and are therefore conventionally referred to as dendritic cell fractions, although these fractions may also include some CD45R/B220 positive B cells (about 20%) and F4/80 positive monocytes/macrophages (about 30%). The monocytes/macrophage cells in the "dendritic" cell fraction were likely to be monocytes, since more adherent mature macrophages were separated out by continuous adherence to plastic. Therefore, the percent transduction of genuine dendritic cells and monocytes in the "dendritic cell" fraction was determined. In addition, since some F4/80 positive cells appeared to be transduced at the injection sites, it was determined if those cells traffic to the spleen. Splenocytes from mice transduced with the β-gal gene were fractionated to obtain "dendritic cell" fractions as described above. Cells were sorted by using two color FACS to collect CD45R/B220 positive, F4/80 positive or double negative cells, *i.e.*, B cells, monocytes or dendritic cells, respectively. Each population was then X-gal stained and β-

gal activities were found in 0.035% of monocytes and 0.025% of dendritic cells, but not in the B cell fractions. These results show that both splenic dendritic cells and monocytes/macrophages from spleen can be transduced and can express vector-encoded proteins.

Since "dendritic cells" from immunized animals were recognized by immune systems and therefore present antigenic peptides, such cells were studied to determine if they can prime naive spleens *in vitro*. One group of mice was immunized to express HIV envelope gp160/120. Their splenocytes were fractionated into three groups: B plus T cells; macrophages; and a "dendritic cell" fraction. Naive litter mates were similarly processed. The resulting six different cell groups were mixed to reconstitute splenocyte populations. Irradiated stimulators were added to initiate *in vitro* CTL cultures. Unfractionated, immunized spleen and splenocytes from mouse immunized with cells expressing HIV envelope gp160/120 were included as controls. The first four combinations, including B plus T cell fractions, from immunized mice were able to induce excellent immune responses. Interestingly, only the "dendritic cell" fractions immunized mice were able to induce immune responses in naive B plus T cell populations, demonstrating that the "dendritic cell" fraction contains antigen presenting cells which can initiate immune responses *in vitro*. As dendritic cells were previously identified as the only population of cells which can initiate immune responses *in vitro*, this "dendritic cell" population must include genuine, antigen presenting dendritic cells.

Since the above data show that *in vivo* transduced dendritic cells and macrophages can induce primary immune responses *in vivo* and *in vitro*, the ability of *ex vivo* transduced cells to stimulate immune responses was also studied. Low density cell fractions were prepared by Percoll gradient separation of naive spleens, followed by stimulation with a cocktail of cytokines to promote cell division. The proliferating cells were transduced with recombinant retroviruses carrying an expression vector encoding HIV gp160/120 using transwell cultures. Aliquots of transduced dendritic and macrophage cells were transferred to naive hosts, followed by *in vitro* restimulation and CTL analysis. As expected, recipient mice induced excellent CTL responses, confirming the role of these cells in immune induction.

To assess the relative efficacy of transduced dendritic cells to induce immune responses by *ex vivo* methods, as compared to other cell types,. transduced dendritic cells were compared to transduced fibroblasts expressing comparable levels of the same antigen injected by identical injection protocol. On the basis of retrovirus-mediated luciferase expression, transduction efficiency of dendritic cells was approximately 0.1% via identical protocol followed by normalization based on protein content. Based on this transduction efficiency, in the adoptive transfer experiments recipient mice were calculated to have received on average about 5×10^2 transduced dendritic cells in a total of 5×10^5 injected cells. The immune response levels induced by these *ex vivo* transduced dendritic cells are comparable to those generated using the same injection protocol to inject 3×10^4 to 1×10^5 fibroblasts expressing the same antigen. On the basis of this calculation, a single transduced dendritic cell induces an immune response equivalent to that generated by 60 to 200 transduced fibroblast cells.

The data above indicate the relative rarity of protein expressing cells. To estimate the frequency of antigen presenting cells among cells expressing the antigen or protein from which it is derived, the following procedures were used to identify these rare antigen presenting cells at the injection sites and in various lymphoid organs.

Ten C57BL/6 mice were injected with an ovalbumin-encoding recombinant retrovirus. Pooled spleens were fractionated into B, T, macrophage and "dendritic cells". These potential antigen presenting cells were incubated overnight with B3.Z cells, CTL hybridomas which express the LacZ protein upon antigenic stimulation. These cultures were stained with X-gal to estimate the level of B3.Z activation. As controls, B3.Z cells were incubated with the following reagents: plate-bound anti-T cell receptor antibodies; a known number of E7.G cells transfected with ovalbumin gene; unimmunized splenocytes; or unfractionated immune splenocytes. A standard curve was generated from the frequency of activated blue B3.Z cells incubated with fixed number of E7.G cells and the frequencies of antigen presenting cells in each splenic fraction were estimated therefrom. An average of about 100 antigen presenting cells per spleen was detected in mice injected with total of 10^7 cfu of the recombinant retrovirus. Pooled splenocytes from these mice

yielded 40% lysis in CTL assays conducted at 100:1 effector to target ratios one week after *in vitro* stimulation.

In another series of experiments, ten C57BL/6 mice were injected with 6×10^7 cfu of the same gene delivery vehicle as used above. Cells were isolated from the injection sites and lymphoid organs. Comparison to the standard curve generated from recognition of E7.G cells revealed that 1×10^5 and 4×10^4 antigen presenting cells were recovered from the inguinal and popiliteal lymph nodes, respectively. 2×10^4 antigen presenting cells were recovered from the injection sites. This data indicates that antigen presenting cells are quite rare. Accordingly, gene therapy-mediated immune induction can be improved by increasing transduction of selective cell types, as discussed below.

As shown above, a hierarchy of cells impacted by gene delivery vehicle-mediated transduction exists. Cells containing the expression vector can be detected by techniques such as PCR in various sources, including whole organs, purified cell populations, or *in situ* from tissue sections. Among cells containing the expression vector (Group I), generally only some will express proteins (Group II), although the relative sizes of the Group I and Group II cell populations may be comparable in many instances. Group II cells an be detected by various techniques, including histochemistry and immunohistochemisty. When duration of heterologous protein expression was examined, no loss of expression occurred during the course of the studies above (10 days), although duration of expression is likely to vary depending on the particular gene delivery vehicle employed.

Antigen processing and presentation involve many steps and parameters: processing of antigenic protein into peptides; transportation of peptide to the endoplasmic reticulum; binding of the peptide to appropriate MHC I class molecules; and microenvironments where peptide-MHC class I complexes can interact with cytotoxic T cells. Thus, only subpopulations of protein expressing cells are likely to present antigenic peptide to the immune system (Group III). The results above confirm this hypothesis, and indeed find that antigen presenting cells are quite rare.

Lastly, a distinct group of cells which do not contain the expression vector and therefore can not express the antigen(s), but nonetheless present antigens (Group IV), exist. Examples of these cells include phagocytic cells which engulf primary transduced

cells or their fragments and reprocess and present antigenic peptides in the context of their own MHC molecules, *i.e.*, cross-priming.

The results above demonstrate that antigen presentation following direct administration of gene delivery vehicles is mediated by splenic dendritic and macrophage cells. Without being bound to a particular theory, it is likely that the cells which are ultimately transduced at the injection site may have been recruited by the inflammatory reaction caused by needle injury. After transduction, the cells are likely to drain via the lymphatics to the spleen and present antigen. *Ex vivo* transduced monocytes/macrophages and dendritic cells are thus excellent sources for transferring immune responses. Therefore, autologous or haplotype-matched, transduced dendritic or monocytes/macrophages may be used as in an immunotherapeutic approach to disease treatment or prevention. However, due to logistical and other complications associated with *ex vivo* gene therapy, directly administering gene delivery vehicles to a patient is preferred. In particularly preferred approaches, *in vivo* transduction of dendritic cells is performed. To enhance such transduction, patients may be treated by pre-injection or co-injection of gene delivery vehicles encoding cytokines such as GM-CSF to selectively cause proliferation of leukocytes. Alternatively, recombinant versions of such immunomodulatory cofactors may be administered. In an especially preferred approach, gene delivery vehicles are targeted to dendritic cells *in vivo*. Production of representative examples of such targeted gene delivery vehicles are described below in Example 2.

Example 2

Murine Dendritic Cells Transduced with Retroviral Vector to Induce Prophylactic and Therapeutic Immune Responses Against Tumor Specific Antigens

1. *Tumor Therapy*

Human papillomavirus (HPV) has been implicated in the genesis of several human cancers, particularly squamous tumors of the cervix and anogenital region. The E7 proteins from some papilloma virus types mediate oncogenic transformation of cervical epithelial cells by binding to the tumor suppressor gene Rb product. The E7 gene is immortalizing and can transform both primary rat cells (Kanda, T. et al., *J. Virol* , 62:610-

613. 1988) and primary human fibroblasts (Watanabe, S, et al. *J. Virol* , 63, 965-969. 1989). Mutational analysis of E7 aimed at elucidating the relationship between structure and function of the protein has revealed that a single point mutation at amino acid 24 removed the transforming ability while the transactivating activity was retained (Edmonds, C. et al., *J. Virology* 63, 2650-2656, 1989) and hence this E7 variant was named p24. Theoretically, E7 protein which is present in the majority of cervical cancers is an attractive target for an *in vivo* stimulated T-cell response.

Since murine cell lines are non-permissive to HPV infections, two surrogate HPV regions were generated by transducing retroviral vectors from the DA/p24 producer line. The DA/p24 producer line was constructed as described in Example 2 above, except that the p24 gene (Edmonds, C. *et al., J. Virology* 63, 2650-2656, 1989) was inserted into the retroviral backbone instead of the *ras* gene. Mammary carcinoma cell line, JC (ATCC# CRL-2116), and colorectal carcinoma cell line, CT26 (Michael Brittain at Baylor College of Medicine) were transduced with DA/p24 to generate JC.p24 and CT26.p24 respectively.

Transduced dendritic cell fractions were prepared as follows. Naive mice were sacrificed and the splenic dendritic cell (SP-DC) fraction was prepared as described in Example 1. Bone marrow dendritic cells (BM-DC) were prepared by flushing the longbones followed by complement mediated elimination of B cells. They were put into RPMI 1640 media containing GM-CSF (500U/ml) and IL-4 (1000U/ml). DA/p24 was seeded in transwells (Corning Costar, Cambridge, MA) with a pore size of 0.45 micron, which does not allow any leakage of producer cells across the membrane. The dendritic cell fraction was added to low adherence tissue culture wells (Corning Costar, Cambridge, MA) and transwells containing DA/p24 were placed above the dendritic cell fraction. After seven days of co-cultivation, the recovered cells were washed three times with PBS before injection into naive recipient mice for prophylaxis or tumor bearing mice for therapy experiments. D2SC/1, a parental immortalized dendritic cell line (WO 94/28113) was transduced with either beta-gal or p24 antigens. The resultant two immortalized dendritic cell lines D2SC/1.beta-gal and D2SC/1.p24, were used as controls.

For therapy experiments, either JC.p24 or CT26.p24 tumor bearing BALB/c mice were treated with syngenic BM-DC or SP-DC dendritic cells transduced by co-cultivation

with DA/p24 producer lines. The tumors were inoculated on day 1 followed by weekly treatment with p24 transduced BM-DC or SP-DC. For immunization experiments, naive BALB/c mice were immunized twice with p24 transduced BM-DC or SP-DC on days 1 and 7, followed by JC.p24 or CT26.p24 challenges on day 14. All mice were measured for tumor volume twice a week. For all experiments, the mean tumor volumes were plotted against days following tumor inoculation.

A. *CT26.p24 Model*

2×10^5 CT26.p24 cells were injected into 50 BALB/c mice (5 groups of 10 mice each) and treated with p24 transduced BM or spleen derived dendritic cells on days 7, 14 and 21. D2SC/1.beta-gal and D2SC/1.p24 were injected at 5×10^6 cells per mouse per therapy. BM-DC and SP-DC were injected at $2\text{-}3 \times 10^6$ cells per mouse or $4\text{-}10 \times 10^5$ cells per mouse respectively, per therapy. Efficiencies of BM-DC and SP-DC were estimated to be 0.1-1% and therefore 2×10^3 to 3×10^4 (BM-DC) or 4×10^2 to 1×10^4 (SP-DC) transduced dendritic cells were believed to be injected per mice per therapy.

The results for the effects of dendritic cell therapy using CT26.p24 cells are shown in Figure 3. Mice immunized with p24 transduced SP-DC or BM-DC, showed less than a 30% decrease in tumor volume when compared to control mice.

B. *JC.p24 Model*

3×10^5 JC.p24 cells were injected into 50 BALB/c mice (5 groups of 10 mice each) and treated with transduced dendritic cells on days 7, 14 and 21. D2SC/1.beta-gal and D2SC/1.p24 were injected at 5×10^6 cells per mouse per therapy. BM-DC and SP-DC were injected at 2-3e6 cells per mouse or $4\text{-}10 \times 10^5$ cells per mouse respectively, per therapy. Transduction efficiency of BM-DC and SP-DC by transwell co-cult was estimated to be 0.1-1% and therefore 2×10^3 to 3×10^4 (BM-DC) or 4×10^2 to 1×10^4 (SP-DC) transduced dendritic cells were injected per mice per immunization.

The results for the effects of dendritic cell therapy using JC.p24 are demonstrated in Figure 4. SP-DC immunized mice displayed approximately a 60% decrease in tumor volume as compared to control animals. Despite the fact that BM-DC vaccinated animals were injected with BM-DC only once, some protection was still observed. Several

animals showed reduction of tumor burdens. D2SC/1.beta-gal immunized mice displayed intermediate levels of protection between unimmunized controls and D2SC/1.p24 immunized animals.

2. *Tumor Prophylaxis*

A. *JC.p24 Tumor*

50 BALB/c mice were immunized twice, on day 1 and 7. 3×10^5 JC.p24 cells were injected into 50 BALB/c mice (5 groups of 10 mice each)) and treated with transduced dendritic cells on days 7, 14 and 21. D2SC/1.beta-gal and D2SC/1.p24 were injected at 5×10^6 cells per mouse per therapy. BM-DC and SP-DC were injected at 2-3 x 10^6 cells per mouse or 4-10 x 10^5 cells per mouse respectively, per therapy. Transduction efficiency of BM-DC and SP-DC by transwell co-cult was estimated to be 0.1-1% and therefore 2×10^3 to 3×10^4 (BM-DC) or 4×10^2 to 1×10^4 (SP-DC) transduced dendritic cells were injected per mice per immunization.

The results for the effects of dendritic cell immunization using JC.p24 are displayed in Figure 5. Despite the fact that BM-DC vaccinated animals were injected only once with BM-DC, some protection was still observed (Figure 6). Several animals showed reduction of tumor burdens. D2SC/1.beta-gal immunized mice (Figure 7) showed intermediate levels of protection between unimmunized controls and D2SC/1.p24 immunized animals.

B. *CT26.p24 Model*

It has been suggested in the literature (Zitvogel, et al., *Exp J. Med.* 183:87, 1996) that dendritic cell based prophylaxis and therapy is more efficacious if the tumor is immunogenic. Dendritic cell prophylaxis or therapy can achieve complete protection or regression of immunogenic tumor whereas only partial protection or regression of weakly immunogenic tumors can be achieved. Since JC.p24 is weakly immunogenic and therefore presumably less responsive to immunotherapy, similar prophylaxis experiments using immunogenic CT26.p24 displayed better protection (see Figure 8).

3. *Comparison to Other Methods of Dendritic Cell Loading*

The following experiment showed that retroviral transduction was equivalent to protein or peptide loading of dendritic cells in inducing immune responses. Beta-gal was used as a surrogate tumor antigen in CT26.beta-gal, CT26 tumors transduced with DA/beta-gal vector. Immune responses were induced by using bone marrow dendritic cells either transduced with beta-gal or loaded with antigenic peptide or protein. The following groups were compared for immune induction and the results showed that the CTL response elicited from all these groups was comparable. This result was significant since dendritic cell loading of peptide or protein were considered to be the best way to elicit immune responses. Similar efficacy was achieved with the use of optimized transduction methods.

1. No treatment
2. BM-DC.beta-gal (1 x 10^6 per mouse) transduction (DA/beta-gal co-cultivation, 7 days)
3. Protein o/n incubation (100 ug/ml)
4. Peptide loading (20 ng/ml) + hb2m (10 ug/ml) for 2 hours (human beta-2 microglobulin is a standard protocol for cell loading of antigenic peptide)

Figure 9 shows the comparison between methods for antigen introduction to dendritic cells. As shown in this figure, equivalent levels of protection for tumor challenges were observed from animals immunized with retroviral vector transduced dendritic cells and dendritic cells loaded with antigenic peptide or protein. Since the molar ratio of beta-gal peptide or protein used to load dendritic cells was in vast excess (>1 million fold), while the retroviral vector transduction efficiency was estimated to be only 1%, it was highly encouraging that the retroviral transduction achieved the equivalent protection to peptide or protein loading.

The following table shows that the level of protein expression required for immune induction by dendritic cell is only 1% of that by fibroblast. Specifically, net CTL lysis, equivalent to fibroblast immunized animals, was observed in mice immunized with transduced dendritic cells with 1/100 of the protein expression. This results demonstrated the superior efficiency of antigen presentation by dendritic cells.

Groups	Relative amounts of beta-gal per immunization	Mean tumor volumes (mm^3) on day 23	Net CTL % lysis at E:T=100:1
No treatment	0	773±173	0
Beta-gal transduced dendritic cells	1	274±50	30
D2SC.1/beta-gal immortalized dendritic cells	5	182+64	95
BC/beta-gal	100	not available	51

While the present invention has been described above both generally and in terms of preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art in light of the description, *supra*. Therefore, it is intended that the appended claims cover all such variations coming within the scope of the invention as claimed.

Additionally, the publications, patents, patent applications, and other materials cited to illuminate the background of the invention, and in particular, to provide additional details concerning its practice as described in the detailed description and examples, are hereby incorporated by reference in their entirety.

We Claim:

1. A gene delivery vehicle comprising a dendritic cell targeting element and an expression vector which directs expression of at least one disease associated antigen.

2. A gene delivery vehicle according to claim 1, wherein the expression vector is carried by a recombinant virus.

3. A gene delivery vehicle according to claim 2, wherein the recombinant virus is a recombinant retrovirus.

4. A gene delivery vehicle according to claim 2, wherein the recombinant virus is an alphavirus.

5. A gene delivery vehicle according to claim 1, wherein the expression vector directs expression of an antigen associated with a disease selected from the group consisting of cancer, heart disease, a bacterial infection, a parasitic infection, and a viral infection.

6. A pharmaceutical composition comprising a gene delivery vehicle according to claims 1-5 and a pharmaceutically acceptable carrier.

7. An *in vivo* method of producing a genetically modified dendritic cell, the method comprising administering to an animal a gene delivery vehicle targeted to a dendritic cell, wherein the gene delivery vehicle comprises a dendritic cell targeting element and an expression vector which directs expression of at least one disease associated antigen.

8. A method according to claim 7, wherein the gene delivery vehicle is a recombinant virus.

9. A method of stimulating a prophylactic immune response in an animal, the method comprising administering to the animal a prophylactically effective amount of a gene delivery vehicle comprising a dendritic cell targeting element and an expression vector which directs expression of at least one disease associated antigen.

10. A method according to claim 9, wherein the gene delivery vehicle is a recombinant virus.

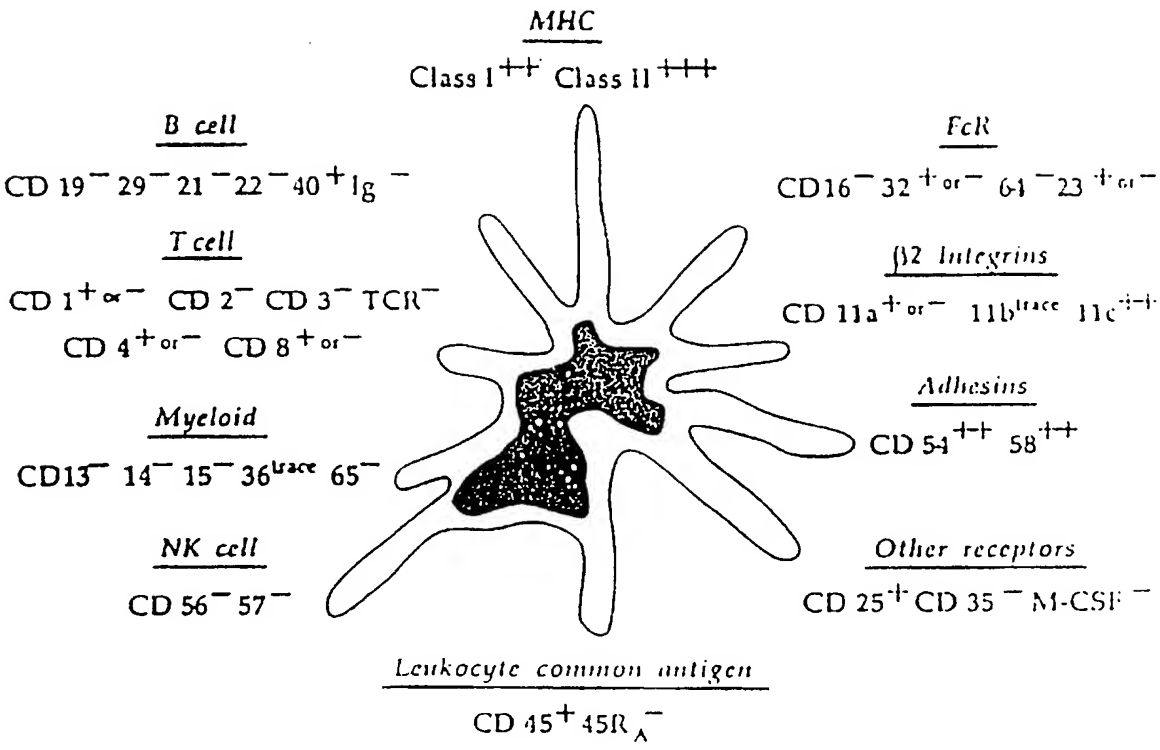

MHC
Class I++ Class II+++
B cell
CD 19− 29− 21− 22− 40+ Ig −
T cell
CD 1+or− CD 2− CD 3− TCR−
CD 4+or− CD 8+or−
Myeloid
CD13− 14− 15− 36trace 65−
NK cell
CD 56− 57−
FcR
CD16− 32+or− 64−23+or−
β2 Integrins
CD 11a+or− 11btrace 11c++
Adhesins
CD 54++ 58++
Other receptors
CD 25+ CD 35 − M-CSF −
Leukocyte common antigen
CD 45+ 45RA−

FIGURE 1

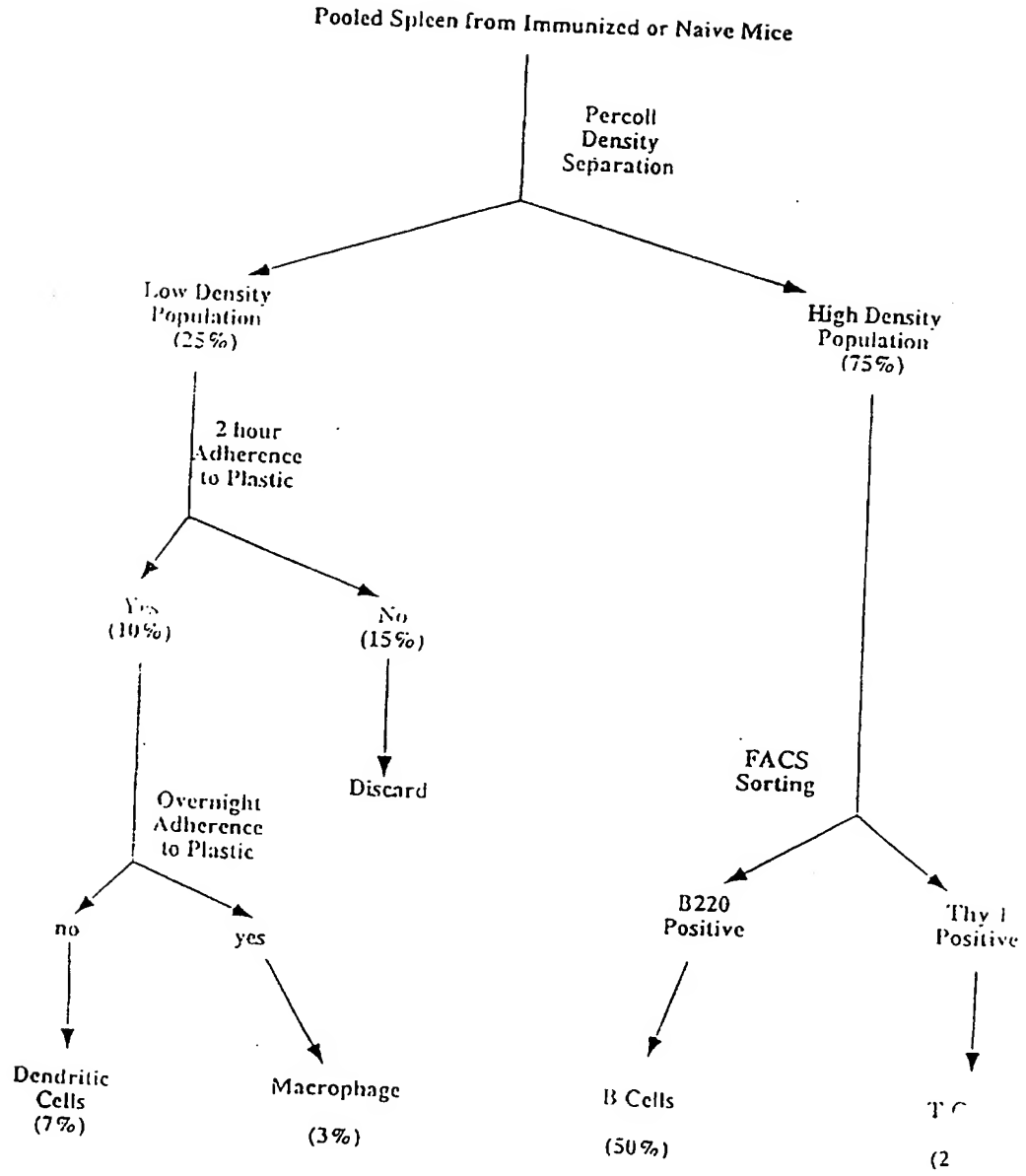
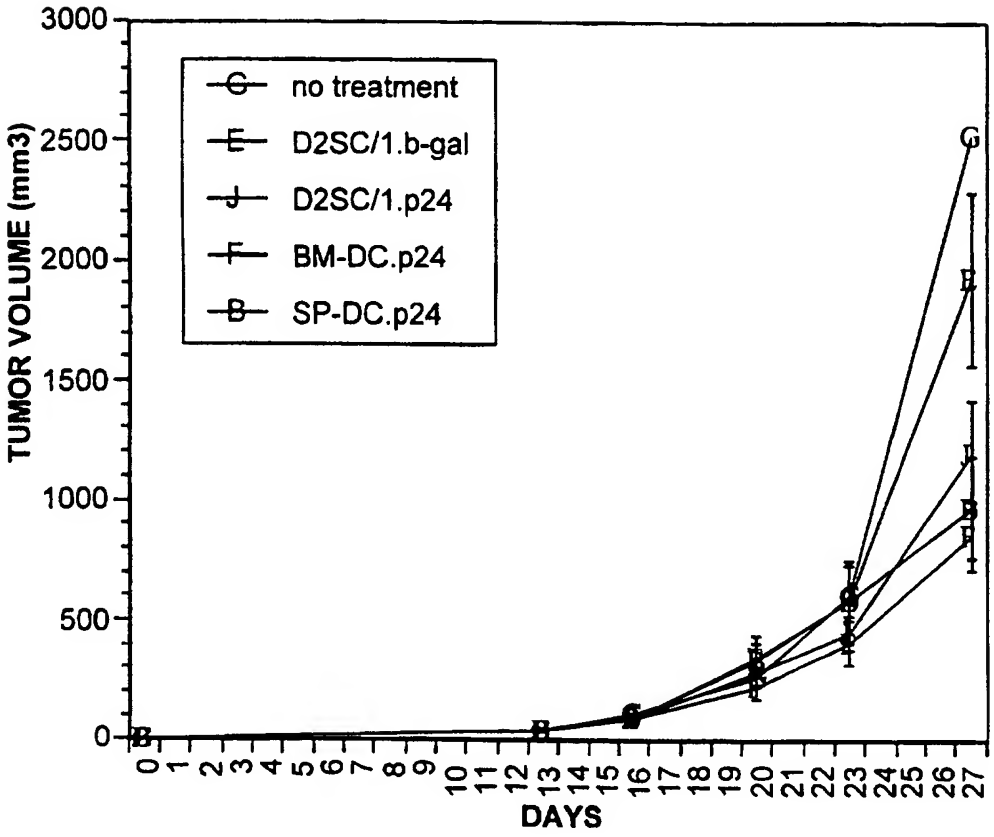

Pooled Spleen from Immunized or Naive Mice
Percoll Density Separation
Low Density Population (25%)
High Density Population (75%)
2 hour Adherence to Plastic
Yes (10%)
No (15%)
Discard
Overnight Adherence to Plastic
no
yes
Dendritic Cells (7%)
Macrophage (3%)
FACS Sorting
B220 Positive
Thy 1 Positive
B Cells (50%)
(2

FIGURE 2

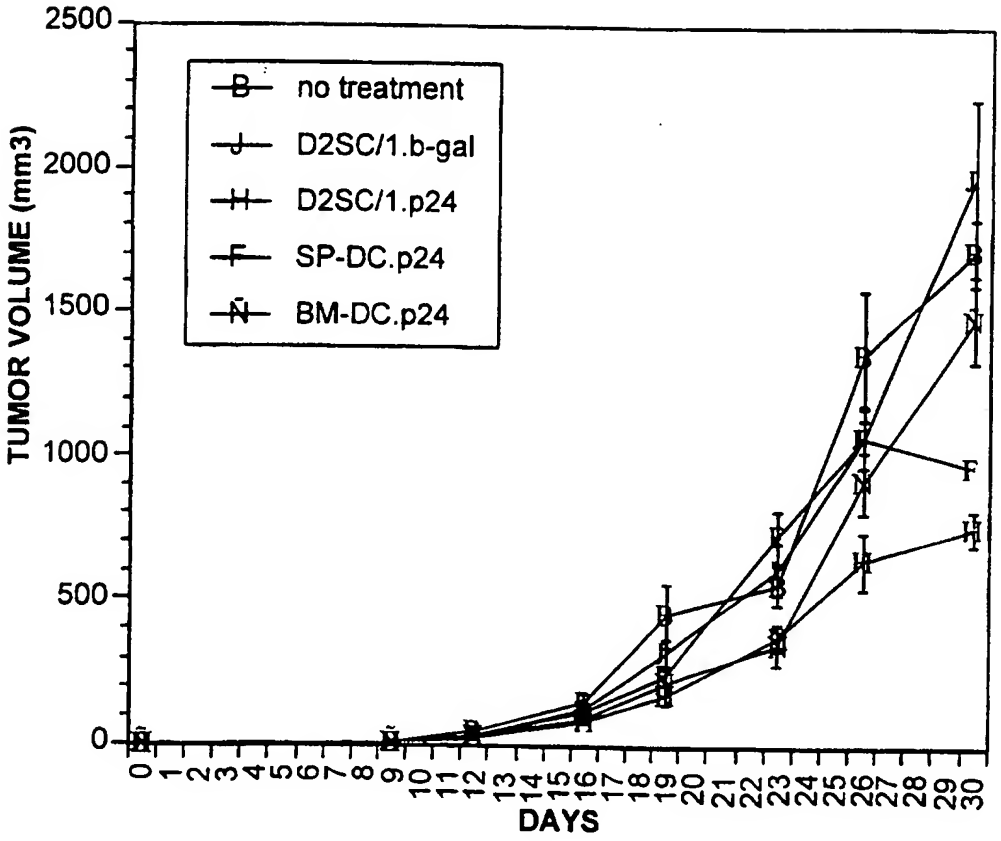
Effect of Dendritic cell therapy on CT26.p24



Balb/c mice were injected with total of 2e5 tumor cells (08/08/96). On Day 8, 15 and 22 (8/15, 8/22 and 8/29) animals were given above treatments . Mice were measured twice a week.

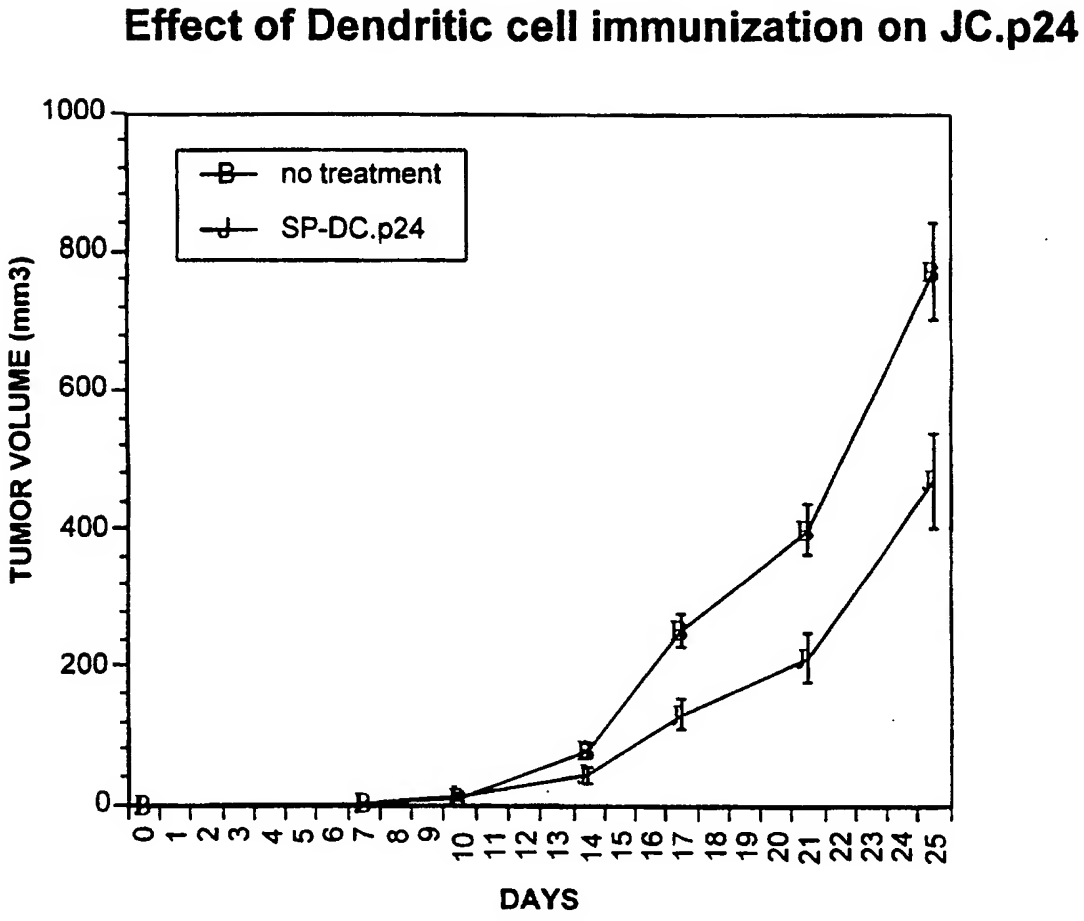
FIGURE 3

Effect of Dendritic cell therapy on JC.p24



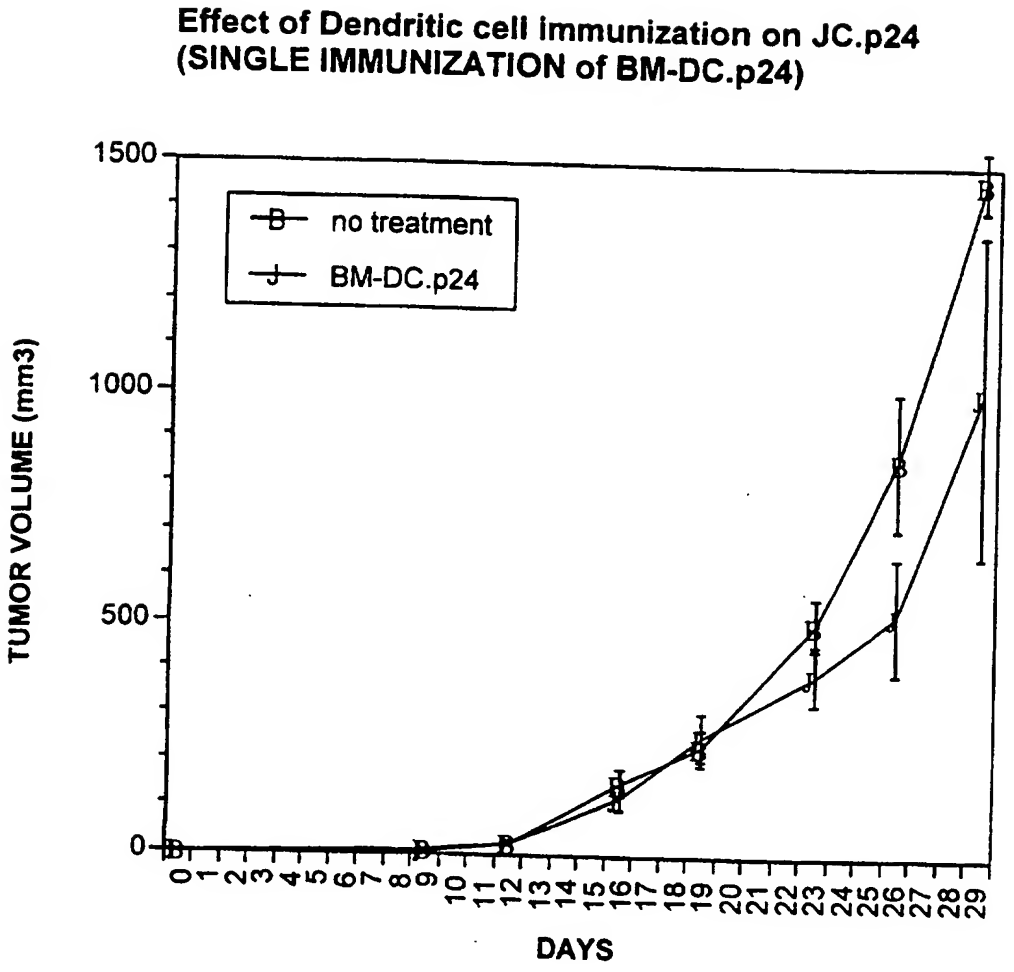
Balb/c mice were injected with total of 3e5 tumor cells (07/08/96). On Day 8, 15 and 22 (7/15, 7/22 and 7/29) animals were given above treatments . Mice were measured twice a week.

FIGURE 4



Balb/c mice were immunized with splenic dendritic cells transduced with p24 twice (06/19/96 and 06/26/96) followed by injection of 3e5 JC.p24tumor cells (07/03/96). Mice were measured twice a week.

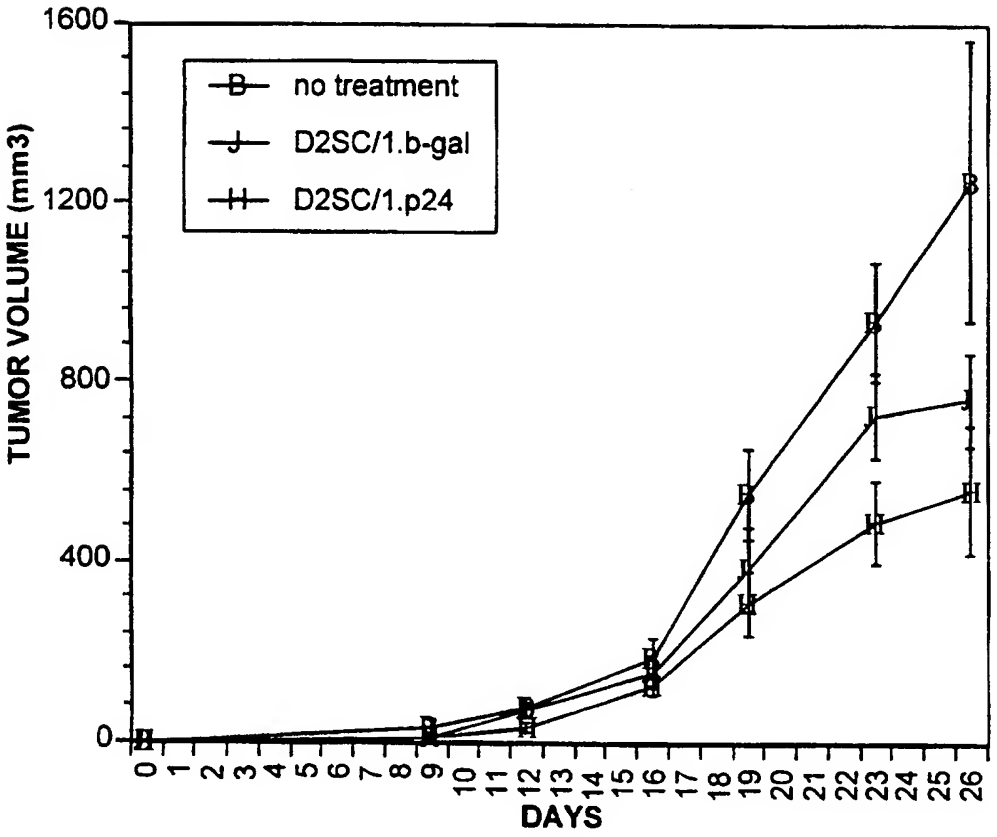
FIGURE 5



BALB/c mice were given above treatments once on day 1 (7/29/96). They were injected with total of3e5 tumor cells (08/5/96). Mice were measured twice a week.

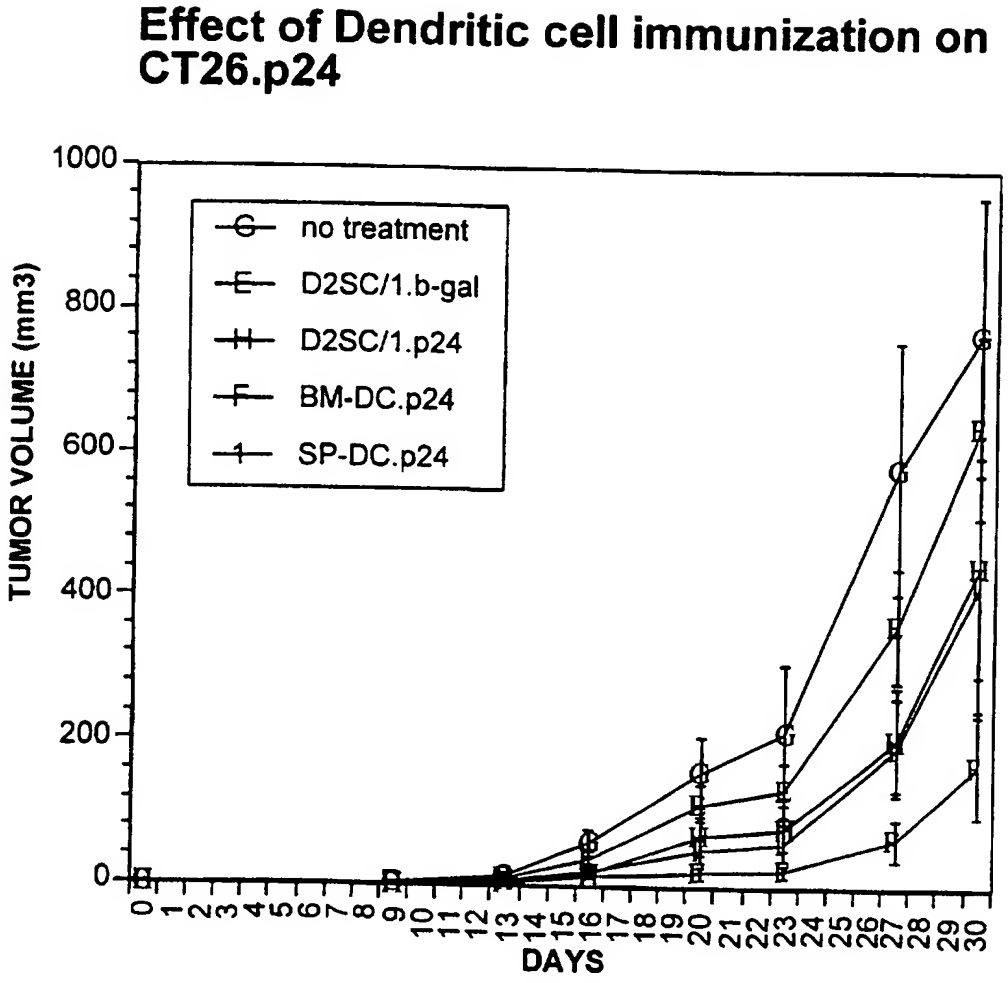
FIGURE 6

Effect of Dendritic cell immunization on JC.p24



BALB/c mice were given above treatments on days 1 and 8 (7/29/96 and 08/05/96). They were injected with total of3e5 tumor cells (08/12/96). Mice were measured twice a week.

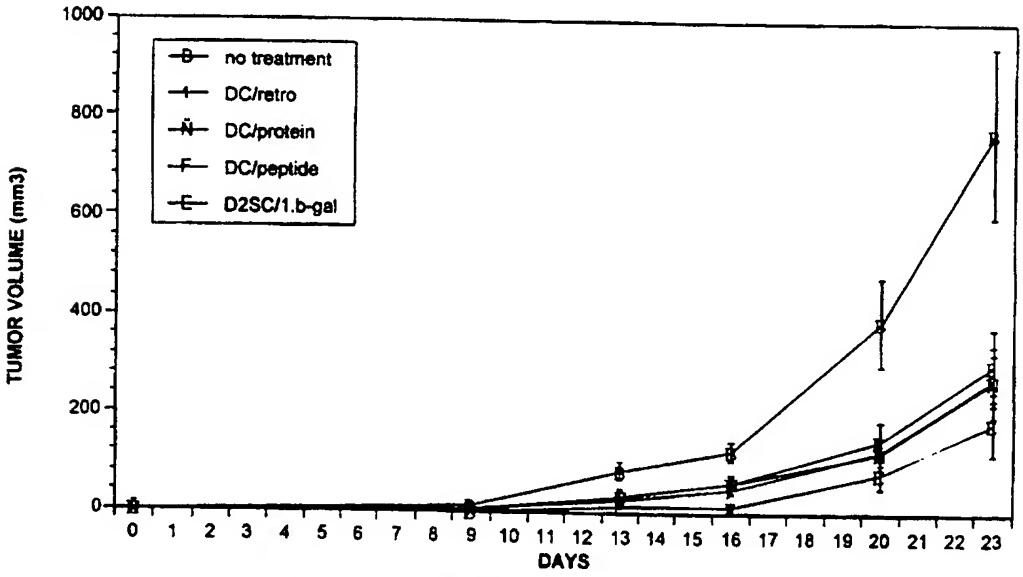
FIGURE 7



Balb/c mice were immunized with above vaccines on days 1 and 7 (08/15/96 and 08/22/96). On Day 15 (8/29/96) animals were injected with total of 2e5 tumor cells. Mice were measured twice a week.

FIGURE 8

COMPARISIONS BETWEEN METHODS FOR ANTIGEN INTRODUCTION TO DENDRITIC CELLS : RETROVIRAL VECTOR, PROTEIN VS. PEPTIDE



Balb/c mice were immunized with above treatement on days 1 and 7 (7/25/96 and 8/5/96). On Day 8, CT26.b-gal tumors (2e5) were injected. Mice were measured twice a week.

FIGURE 9

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/20105

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/63 C12N15/86 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENE THER. MEETING COLD SPRING HARBOR, 1994, page 25 XP000601252 BUELER H ET AL: "RETROVIRUS-TRANSDUCED ANTIGEN PRESENTING CELLS FOR THE SPECIFIC IMMUNOTHERAPY OF CANCER" see the whole document ---	1-10
A	JOURNAL OF VIROLOGY, vol. 69, no. 4, April 1995, pages 2357-2365, XP002014861 BAIER ET AL: "IMMUNOGENIC TARGETING OF RECOMBINANT PEPTIDE VACCINES TO HUMAN ANTIGEN-PRESENTING CELLS BY CHIMERIC ANTI-HLA-DR AND ANTI-SURFACE IMMUNOGLOBULIN D ANTIBODY FAB FRAGMENTS IN VITRO" see the whole document ---	

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
12 May 1997	20. 05. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Sitch, W

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/20105

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 96157235, BALTZ: "VACCINES IN THE TREATMENT OF CANCER" XP002030724 see abstract & AMERICAN JOURNAL OF HEALTH-SYSTEM PHARMACY, vol. 52, no. 22, 15 November 1995, pages 2574-2585, ---	
A	WO 95 05853 A (UNIV CALIFORNIA ;CARSON DENNIS A (US); RAZ EYAL (US); HOWELL MERED) 2 March 1995 see page 14, line 14 - page 22, line 20 ---	
A	EP 0 245 078 A (CONNAUGHT LAB) 11 November 1987 see page 3, line 19 - line 27 ---	
A	DATABASE MEDLINE FILE SERVER STN KARLSRUHE ABSTRACT 95169512, WANG ET AL: "DNA INOCULATION INDUCES PROTECTIVE IN VIVO IMMUNE RESPONSES AGAINST CELLULAR CHALLENGE WITH HIV-1 ANTIGEN-EXPRESSING CELLS" XP002030725 see abstract & AIDS RESEARCH AND HUMAN RETROVIRUSES, vol. 10, no. S2, 1994, pages S35-S41, ---	
A	WO 93 10814 A (VIAGENE INC) 10 June 1993 cited in the application see page 2, line 35 - page 4, line 12 -----	

1

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US 96/20105

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 7-10
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 7 to 10 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inte: onal Application No
PCT/US 96/20105

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0714308 A	05-06-96
		JP 9501936 T	25-02-97
EP 0245078 A	11-11-87	CA 1327523 A	08-03-94
		DE 3775458 A	06-02-92
		JP 6074210 B	21-09-94
		JP 63045228 A	26-02-88
		US 4950480 A	21-08-90
		US 5194254 A	16-03-93
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		EP 0615453 A	21-09-94
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		JP 7501942 T	02-03-95
		NO 941986 A	27-07-94

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